

STUDIES ON LEUCOCYTIC ASCORBIC ACID
AND THE IMPORTANCE OF
VITAMIN C IN POST-SURGICAL PATIENTS

by

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GENERAL INTRODUCTION

General Introduction

Historical Investigations

The clinical condition called scurvy was known to Hippocrates, who wrote, about 400 B.C., "those who labour under that disease sometimes have ulcers on their legs while others (healed ulcers) break out anew". (Stewart & Guthrie, 1953). Hippocrates thus recorded an occurrence which was to be repeated countless times in the centuries following, particularly on long sea voyages, or on military operations like the Crusades (Van Wersch, 1954), namely that in scorbutic, or near-scorbutic states, healed wounds broke down, while new wounds failed to heal.

Over two thousand years after Hippocrates, James Lind in his brilliant "Treatise on Scurvy" (1753) quoted the following classical description of wound deterioration in the presence of frank scurvy, from the pages of a Mr. Ive's Journal (1742): "John Thomas, marine, on the 18th August 1742, got, by a musket ball from the Spaniards, a very bad fracture of the Os humeri. By the end of November a union was brought about and a sound skin brought over the incisions. His supply of fresh provisions was stopt upon which he fell into a bad scurvy, the first symptom of which that appeared was the breaking out of the late wounds in his arm."

Thus, two hundred years ago it had not only been observed that scurvy resulted in healed wounds re-opening,

but it had also been clearly stated by Bachstrom in 1734 (Stewart and Guthrie, 1953) and noted by Lind, that scurvy was due primarily to a lack of fresh vegetables. Bachstrom coined the word antiscorbutic, which suggests the essentially preventive nature of its action. Lind's recommendations on nutrition to the Royal Navy adopted in 1795 can be seen, in retrospect, to be a fine example of the application of preventive medicine, but not surprisingly, the negative cause of disease was not understood by the contemporary mind.

The rapid growth of bacteriology in the 19th century rooted the concept of positive causation of disease still deeper in the minds of men. As recently as 1913, Scott's Antarctic expedition carried rations virtually devoid of any dietary antiscorbutic, demonstrating that even then the accepted curative action of the antiscorbutic principle had not been logically extended to include a preventive role.

Concurrent with the tragic death of Captain Scott, the findings of Sir Gowland Hopkins on accessory food factors were in dispute in scientific circles. These findings were originally published in 1912, but only by 1923 were Hopkins' principles widely accepted, so that Cherry-Garrard (1952), describing the familiar scorbutic wound breakdown on Scott's expedition, suggests on reflection that "the absence of certain vitamins must have had a great

influence upon the fate of the polar party."

With the acceptance of Hopkins' discovery of accessory food factors, the modern era of scientific investigation into the antiscorbutic principle begins, and this will now be briefly reviewed, with particular reference to wound healing. Before doing so, however, it is pertinent to mention that, at the time modern scientific investigations began, there was an important lesson to be learned from earlier work. Superimposed on the nutritional factor, the importance of which Lind had emphasised, there was a physiological stress factor which, if operating, could precipitate the appearance of scurvy and concomitant symptoms. In these facts we have the elements of the present thesis, that the nutritional requirement of the antiscorbutic factor is increased by the trauma of surgery.

Modern Investigations

Events in the Healing of Wounds

The importance of cellular migration into the wounded area followed by a phase of mitotic activity was established by the work of Arey (1932), and of Young, Fisher and Young (1941). The latter authors also showed that secondary wounds had a faster rate of healing than primary wounds and deduced that some accessory accelerating factor must operate in the healing of secondary wounds which is lacking in an initial injury.

The first studies of the relationship between scurvy and the failure of wound healing are those of Aschoff and Koch (1919), Hojer (1924) and Wolbach and Howe (1926). These histological investigations showed that in the absence of the antiscorbutic factor, the function of the mesenchymal cells was disturbed and suggested that the nature of this disturbance was the defective formation of intercellular matrix.

The application of tensiometry to wound healing studies was made by Howes, Sooy and Harrey (1929) who showed that no cohesive force could be recorded in a newly inflicted wound for three or four days, but between the fifth and twelfth day, there was a rapid gain in tensile strength. By plotting tensile strength values against time, they showed that the resulting curve was sigmoid in shape and pointed out that the initial lag phase corresponded to the period of traumatic inflammation. They suggested that during this period, the wound was being prepared for healing by removal of dead material by phagocytosis. This work was confirmed by Sandblom (1944), who later showed that the so-called lag-phase persisted in the scorbutic animal and that the period of gain in tensile strength did not occur.

Hunt (1941) was able to show that in near-scurvy states in the guinea pig, the collagen of a recently healed wound reverted to a pre-collagen condition, as evidenced by

a change in histological staining properties and with consequent diminution in tensile strength. This observation could possibly explain the easy rupture of wounds in scorbutic subjects.

The increased susceptibility to infection often seen in scurvy is possibly due to the direct relationship which was shown to exist between the vitamin C content of the leucocytes and their phagocytic activity (Lawryniewicz, 1931; Cottingham and Mills, 1943; Nungester and Ames, 1948).

The soundness of the classic investigation by Wolbach and Howe (1926) is shown by the fact that the defective function of the four types of mesenchymal cells (odontoblasts, osteoblasts, chondroblasts, and fibroblasts) can explain all the observed pathological lesions in scurvy except the deterioration in the integrity of the vascular elements. The greater the activity of the mesenchymal cells, the more obvious are the changes when these activities slow down or cease as in scurvy. (Wolbach, 1953)

The work of Lee (1960) has thrown some light on the role of vitamin C in vascular physiology. Using micro-manipulative procedures, he has shown that vitamin C is necessary to maintain responsiveness to stimulation with epinephrine and hence to maintain the animal's total vasocompensatory reaction to vascular stress as in haemorrhage. A further point worth noting from Lee's work is that

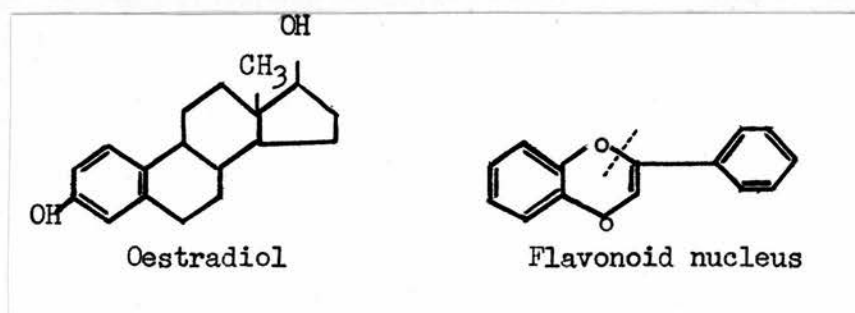
he found the location of the petechiae occurring in vitamin C deficiency was almost completely in the collecting venular system, and he points out that in this portion of the peripheral vascular bed, the smooth muscle cells of the venules are discontinuous, with areas of the vessel wall consisting only of endothelium, ground substance and collagen fibrils.

The degradation of collagen consequent on vitamin C deprivation may occur in collagenous tissues (Robertson, 1961; Gould, 1960) but earlier radioisotope studies (Neuberger and Slack, 1953) had led to the conclusion that collagen once laid down, was metabolically inert. Hence it is uncertain if the haemorrhagic tendency in scorbutic states is due primarily to a reduced number of collagen fibrils in the walls of the venules. Lee (1960) has suggested that the dilated peripheral vessel seen in hypovitaminosis C is particularly liable to rupture because it is distended, rather than because of a structural defect in the mural elements.

Some authors have found that both vitamin C and the bioflavonoids are essential for the maintenance of the intact capillary wall and that without either of them, bleeding can easily occur. (Robson and Duthie, 1950; Martin, 1955). Martin, Moss and Beiller (1955) demonstrated a striking synergism, in vivo, between the bioflavonoids and

vitamin C.

The maintenance of vascular integrity was investigated indirectly by Clemetson, Blair and Brown (1962) who studied the factors initiating menstrual bleeding. They showed that corresponding decreases in capillary strength occurred when oestrogen levels were falling both after the ovulation peak and prior to menstruation. Clemetson et al point out that the petechial haemorrhages seen in senile vaginitis respond well to oestrogen therapy or to vitamin C supplements and postulate that oestrogens and possibly other steroids enter into competition with the bioflavonoids for a substrate in the capillary wall, where they maintain integrity until such time as they are metabolised or withdrawn. The structural similarity between the oestrogens and the flavonoid nucleus is shown below.



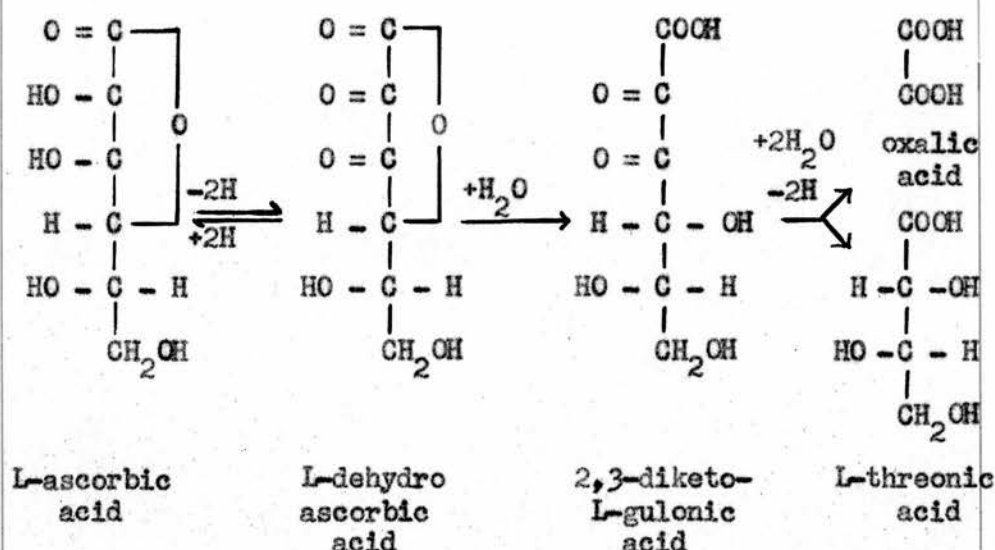
The physiological activity of the bioflavonoids has been ascribed to the tendency of the flavonoid nucleus to split between the ring oxygen and the 2-carbon position (shown in the diagram) resulting in the formation of chalcones. The latter compounds, like vitamin C, have strong reducing properties and they revert to the original cyclic form.

The role played by vitamin C in the prevention of scurvy can thus be seen to be a complex one, in which normal functional activity of mesenchymal cells and leucocytic phagocytosis continue, as well as vascular integrity and maximum vasocompensatory reaction to epinephrine.

Chemical Investigations:

The regeneration of new tissues must ultimately be a matter of the synthesis, at the molecular level, of chemical substances in the wound. We therefore find that with the isolation of vitamin C in 1928 by Szent-Gyorgi from adrenal tissue, and four years later by King and Waugh (1932) from lemon juice, the problem of unravelling the antiscorbutic-deficiency complex became primarily a biochemical one. The elucidation of the chemical structure of vitamin C by Haworth, Herbert, Hirst and Percival (1933) gave further impetus to the new biochemical approach.

Herbert, Hirst, Percival, Reynolds and Smith (1933) made an exhaustive study of the chemical properties of vitamin C and showed that the following scheme represents the usual route of spontaneous decomposition of the vitamin in aqueous solution, particularly above pH 7.



The primary oxidation product of ascorbic acid is dehydroascorbic acid which is unionised, having lost the acidic ene-diol grouping. This oxidation is reversible and so dehydroascorbic acid is also physiologically active, but the opening of the lactone ring to produce 2,3 diketo-L-gulonic acid results in a loss of biological activity.

The first chemical synthesis of vitamin C was carried out almost simultaneously by the groups headed by Reichstein (1933) and Hirst (1933).

Biochemical Investigations:

The discovery of the almost unique occurrence of hydroxyproline in collagen provided a convenient means for the chemical estimation of collagen synthesis. (Neuman and Logan, 1950). Gould and Woessner (1957) and Gould (1958) used polyvinyl sponges implanted subcutaneously in guinea

pigs. They were able to show that in scorbutic states, the inability to synthesise collagen as measured by the hydroxyproline content of the implanted sponges, could be abolished within 24 hours of giving ascorbic acid. Robertson and Schwartz (1953) used another technique in the study of collagen synthesis, that of carrageenin granuloma formation. Using C^{14} proline, they showed that the source of hydroxyproline for collagen synthesis was proline, and that the hydroxylation reaction appeared to be defective in vitamin C deficient guinea pigs. Mitoma and Smith (1960) contested this view, since urinary excretion of hydroxyproline continued in scurvy, but they were unable to substantiate their counter-claim that all protein synthesis was defective in scurvy.

The studies of Dunphy and Udupa (1955) and of Dunphy, Udupa and Edwards (1956) into the sequential biochemical processes in wound healing confirmed the two-phase findings of previous studies by microscopy, tensiometry and histology. The first phase lasted 3 - 5 days after wounding, and was marked by increasing concentrations of mucopolysaccharides, measured as hexosamine. Migration of fibroblasts into the area was observable from the time of wounding but increased markedly after several days and with the invasion by capillaries, collagen synthesis proceeded. Simultaneously a sharp decline in hexosamine concentration occurred. On the twelfth day the collagen content of the wound had

reached a maximum value, as had the tensile strength. It can be seen from this study that the "lag phase" of earlier workers is a period of complex events related to later collagen synthesis. By applying these studies to scorbutic animals, Dunphy et al. (1956) were able to show that the first phase of mucopolysaccharide synthesis was unimpaired but greatly extended, and that no second phase of collagen synthesis followed. There was abundance of fibroblasts but these were not mature and no hydroxyproline could be demonstrated. If ascorbic acid was given to such animals, there was a sharp fall in hexosamine content and a sharp rise in collagen synthesis, both of which were detectable within 12 hours.

Evidence has also been presented to suggest that, although mucopolysaccharide synthesis as measured by hexosamine concentration proceeds normally in the scorbutic guinea pig, the sulphation of these compounds is defective (Upton & Odell, 1956). These authors found that the uptake by scorbutic granulation tissue of S^{35} , given in the form of sodium sulphate, was much less than in the tissue of a control group of normal animals, and both Reddi and Norstrom (1954) and Kodicek and Loewi (1956) found that the incorporation of radio sulphate into the chondroitin sulphate of granulation tissue was impaired in scorbutic guinea pigs.

The fact that leucocytes are also involved in the

changes in sulphur metabolism concurrent with wound healing has been shown by Williamson, Whalen and Haley (1962) who found that surgical injury had no effect on the ability of the white cells to incorporate methionine, but that the uptake of L-cystine is increased markedly. This increase is maximal after three days, but is still apparent after seven days. The full significance of these findings must await the elucidation of the biochemistry of the mucopolysaccharides and sialic acids, a task which Gottschalk (1960) has already begun.

The question of the need for fibroblasts for the production of collagen was clarified by the work of Stearns (1940) and Porter and Vanamee (1949). The latter authors, using the technique of electron microscopy, demonstrated an interaction between fibres laid down on either side of the cellular membrane and materials in the ground substance. Highberger and Gross (1951) showed by in vitro studies, that a dialysed acidic solution of collagen can be made to precipitate into a collagen fibre in the presence of an acid polysaccharide such as chondroitin sulphuric acid. The suggestion was made by Meyer (1947) that the young fibroblasts, having invaded the wound area, secrete hyaluronic acid, and that ascorbic acid is needed as a non-enzyme catalyst for the replacement of hyalonic acid by chondroitin sulphate and collagen. It is interesting to note

that monodehydroascorbic acid, which is known to occur in vivo (Yamazaki, Mason and Piette, 1960), can depolymerise hyaluronic acid (Robertson, Ropes and Bauer, 1941).

Possible Functions of Leucocyte and Platelet Ascorbic Acid.

Roos (1957) suggested a process of continuous intravascular blood coagulation, which would explain the normal maintenance of haemostasis and the continuous utilisation of the various clotting factors. The platelets are an important component of the haemostatic mechanism and, if their mode of action involves their deposition on the vascular membranes, this would constitute a means of transporting vitamin C to sites where it is believed to function in the synthesis and maintenance of ground substance (Penney and Balfour, 1949).

In the same way, the leucocyte may be the means whereby vitamin C is transported to areas within a wound before vascularisation can supply this need. Since the inflammatory reaction, which develops after wounding, results in an increased metabolism in the area before growth of the capillaries has taken place, the transport of vitamin C to such areas by leucocytes may be an important prelude to wound healing. Of interest here is the possibility that the polymorphonuclear leucocyte can alter to become a fibroblast. Allgower (1956) has shown in tissue culture that this type of leucocyte can assume elongated

cytoplasmic processes, thus morphologically resembling a fibroblast, while Hisoda and Keio (1960) have observed that most of the cells seen in wound tissue have the appearance of leucocytes or fibroblasts. Also, it is well known that the polymorph can migrate from the blood stream through intact cell membranes by amoeboid movement. The leucocyte is therefore admirably suited to fulfil the role of emergency transporter of vitamin C, although other reports indicate that the cells in regenerating wound tissue originate from areas of fixed connective tissue cells adjacent to the adventitia of arteries and veins. (McDonald, 1959).

It has often been suggested that vitamin C may act in a respiratory capacity. R ih  (1958) proposed such a function after showing that, both in the guinea pig and man, dehydroascorbic acid rapidly passes across the placenta from mother to foetus, where it is found in the reduced condition, whereas ascorbic acid cannot cross the placental barrier. It has been known for some time that foetal blood has high vitamin C content, even in cases where the maternal blood and tissue concentrations are low, and also that the first urine passed after birth contains a high concentration of the vitamin, suggesting a decreased requirement simultaneous with the assumption of independent life (Ingalls, 1938; Hamill, Munks and Williams, 1947). When these events are viewed in the knowledge that the foetus spends the latter part of intra-

uterine life in a state of physiological hypoxia, (Barcroft, 1946) it is tempting to speculate on a respiratory function for the vitamin in states of low oxygen tension.

Two observations can be cited in support of this suggestion. Galperina (1951) has shown that the introduction of ascorbic acid into the rabbit foetus, or the mother, leads to a prolongation of life of the foetus after tying off the umbilical cord. Secondly, Krauss, Cilley and Farmer (1950) found that repeated exposure of human subjects to a rarified atmosphere at 18,000 ft., without supplemental oxygen, resulted in a retention or utilization of the vitamin, shown by a rapid and sustained drop in their vitamin C excretion. These findings suggest that more vitamin C is required in hypoxic states, and that it functions when the metabolism of the organism is more anaerobic in character, either generally or locally, as in the avascular regions of a wound.

Some confirmation of this possible anaerobic role of vitamin C has come from the work of Stern and Timonen (1954). When investigating the metabolism of the cell nucleus, they found that the cytochrome and flavoprotein systems were both lacking there and concluded that nuclear metabolism must be anaerobic. They suggested that the function of vitamin C was one of hydrogen transport, and demonstrated a close relationship between ascorbic acid concentration and the

activity of the mitotic process. These observations agree well with those of R  ih   (1958) on the importance of ascorbic acid in the hypoxic foetus, and with the apparent independence of tissue culture cells of vitamin C supplies noted by Gould (1958) and Eagle (1955).

Human Requirements of Vitamin C.

In the foregoing section, the biochemical background of this investigation has been given. While the discovery of the specific biochemical lesion caused by vitamin C deficiency is important, the optimum nutritional requirement of post-surgical patients is the main object of this study. The state of knowledge in this field when these studies began will now be discussed.

The investigation of human minimal and optimal daily requirements has given rise to diverse opinions. There is general agreement that the minimal daily requirement for protection against scurvy is 5 - 10 mg., although Abt, von Schuching and Enns (1963) have recently claimed that the minimum requirement may possibly be as low as 1 - 3 mg./day for man. Their findings were based on calculations of the half-life period of ascorbic acid in a subject on low intake of the vitamin, having first shown that the half-life period varied inversely with the level of intake.

For the optimum requirement, the League of Nations (1938) Technical Commission on Nutrition, suggested 30 mg.

of vitamin C daily, and this has been adopted by the British Medical Association (1950) and by the Medical Research Council (1953). The National Research Council of the United States (1958) however, recommends a daily intake of 70 - 75 mg./day. The higher level of intake recommended by the United States Council is based on tests in humans and animals designed to find the quantity of the vitamin that would produce satisfactory healing of wounds, enzyme action and cell proliferation (Uhl, 1958). Uhl also pointed out that 70 - 75 mg. of vitamin C per day brought the plasma ascorbic acid level up to the concentration found in breast-fed infants, without causing excretion and therefore waste.

In the light of recent research (Martin and Darby, 1957; Plough and Bridgforth, 1960) it would appear that an intake of 30 mg. per day does not allow a margin for tissue reserves, since this level of intake over weekly periods is associated with rapid depletion of body stores (Peters, 1948). In confirmation of this, Steele, Liner, Pierce and Williams, (1955) found that a daily intake of 40 - 50 mg. of the vitamin was necessary to restore the white blood cell to normal in a group of volunteers depleted of the vitamin. With an intake of less than this amount, white blood cell values continued to fall slowly, or remained at the depleted level.

Morse, Potgieter and Walker (1956 a, b) found that,

in women in two age groups (28-34) and (56-77), the white cells of the younger group reached maximum values at a lower daily intake of vitamin C (60 mg.) than the older age group (70-80 mg.), suggesting that the daily requirement might be increased with age, or that intestinal absorption became less efficient.

Recommendations of daily intake apply to subjects who are in a state of normal health. Increased daily requirements have been reported in such varied conditions as pregnancy (Martin and Darby, 1957; Pankamaa and R  ih  , 1957); lactation (Smith, 1938); during growth (Reid, 1948); in infections (Sigal and King, 1937; Abt and Farmer, 1938; Abt, Hardy and Maaske, 1942; Crandon, L  nnihan and Reif, 1961); burns (Emery, Rosen and Levenson, 1960; Taranovich, Glushakova and Laguto, 1961); and wound healing (Bartlett, Jones and Ryan, 1942; Abt, von Schuching and Roe, 1959 a,b; Crandon, Lund and Dill, 1940; von Schuching, Enns and Abt, 1960).

The vitamin C status of any pre-surgical patient may range from clinical scurvy at one end of the scale to tissue saturation at the other. At the present time it would be difficult for the clinical biochemist, if so requested, to determine if the vitamin C reserves of a patient prior to operation were adequate for major surgery, since little is known regarding the change in requirements after surgery.

Patients submitted for either elective or emergency surgery derive from a cross section of the general population of this country. If the incidence of scurvy and sub-clinical scurvy in recent years found in general surveys is considered (Thomson, 1954; Alstead, 1954; McMillan and Inglis, 1944; Cutforth, 1958; Rozner and Lloyd, 1958; Mecray, 1955), it is evident that many patients who undergo surgery have suboptimal nutritional status with reference to vitamin C, which is not consistent with a good prognosis. Crandon et al (1961) believed that the majority of post-surgical patients can be maintained adequately on an intake of 100 - 300 mg. vitamin C per day, but state that, if low tissue levels of the vitamin are present pre-operatively, time must be allowed for the tissue content to build up, since the movement of the vitamin from plasma to cells appears to be a slow process.

In summary, the British Medical Association, and the Medical Research Council have recommended a daily intake of 30 mg. of vitamin C, while the National Research Council of the United States has recommended the higher intake of 70 - 75 mg. Both these figures apply to persons in normal health. There is evidence from nutritional surveys that few persons enjoy optimal nutrition with reference to vitamin C, and that the post-surgical patient has an increased requirement for this vitamin.

EXPERIMENTAL SECTION

Experimental Section*

Introduction

Delineation of the Problem

On the basis of the two phase nature of the healing process, previously discussed, (Dunphy and Udupa, 1955) it was decided to restrict the biochemical observations in the present studies to two periods of time in the post-surgical patient. The first would be within the period from surgery to 3 days later, and the second from the 3rd - 7th post-operative day. It was thought that the metabolic need for vitamin C should be investigated in both periods since biochemical abnormalities in the scorbutic animal have been demonstrated in each one. (Upton and Odel, 1956; Dunphy, Udupa and Edwards, 1956).

When these studies commenced in 1960, claims that there were increased requirements for vitamin C in post-operative patients, or in wound healing experiments, were

* Throughout the experimental and succeeding sections it has been found convenient to use the following abbreviations with the meanings shown:-

AA	L-Ascorbic acid
DHA	L-Dehydroascorbic acid
DGA	2,3-Diketogulonic acid
TAA	Total ascorbic acid (By this is meant the sum of AA, DHA and DGA.)
CMB	p-chloromercuribenzoic acid

based on (1) the finding of lower whole blood, plasma or buffy layer concentrations of the vitamin in these cases than in normal persons or control animals; (2) observations made on patients following severe injury, who exhibited selective concentration of the vitamin in the traumatised area (Bartlett et al, 1942; Posthlewthwait, Chen and Kamin, 1960; Schauble, Chen and Dillon, 1960), or in whom a need could be shown for greatly increased oral dosages to attain body saturation, (Lund, Levenson, Green and Robinson, 1947; Andrease and Brown, 1946). In some cases, the healing of associated wounds was delayed, suggesting to Levenson, Upjohn, Preston and Steer (1957) that "the seriously injured human behaves physiologically like the scorbutic".

The present study aimed to investigate the validity of these claims and Douglas (1963) reflected the contemporary surgical outlook when he stated that an investigation of leucocyte vitamin C in surgical patients, with a view to discovering the prevalence of deficiency states, would be an interesting study.

It was against this clinical background that the present work was performed and consideration given to a means of demonstrating an increased metabolic requirement for the vitamin post-surgically. A decreased plasma or leucocytic concentration compared to the pre-operative value was considered as a possible index.

Choice of Index.

Plasma is not an ideal medium in which to detect small decreases in vitamin C concentration since the normal level is already low.

The buffy layer of human blood contains a much higher concentration of the vitamin (Stephen and Hawley, 1936; Cuttle, 1938; Butler and Cushman, 1940) than that in plasma. The latter authors suggested that, because of this higher concentration (22 - 36 mg./100 ml.), the leucocyte level would reflect any deficiency of the vitamin much more accurately than that in the fasting plasma (0.2 - 2.5 mg./100 ml.). This suggestion was confirmed by Bessey, Lowry and Brock (1947), Morse et al (1956 a, b) Steel et al (1955), and by Chevillard and Hamon (1943). The last-named workers showed that the vitamin C content of leucocytes and platelets closely paralleled that of the body tissues generally, thus confirming the earlier experiments of Crandon et al (1940). In these studies on human scurvy, Crandon had demonstrated that the appearance of petechiae associated with vitamin C deficiency became apparent only after the vitamin had been undetectable in the plasma for over three months, whereas the white cell level reached zero values at approximately the same time as the scorbutic lesions appeared. The leucocyte concentration was therefore chosen as the parameter with reference to which the study of the

somatic reserves of the vitamin would be made in surgical patients.

Problems of the leucocytic metabolism of vitamin C

A second aspect of the problem also concerned with the leucocyte was suggested by the work of Crandon, Mikal and Landau (1952). These authors had claimed that in the post-surgical patient, particularly when seriously ill, vitamin C serum levels did not correlate well with those of the leucocytes and that the predictable relationships between these measurements, seen in deprivation experiments, did not always hold good in traumatic conditions. In an attempt to clarify this problem of the variable distribution of vitamin C, the metabolism of the oxidised and reduced forms of the vitamin with reference to the leucocyte was investigated.

A further justification for this particular study derived from the fact that the published evidence for the role of the white blood cell in the metabolism of vitamin C was conflicting. Lloyd (1951), had suggested that leucocytes oxidise AA to DHA. On the other hand, the erythrocyte absorbs and reduces DHA rapidly (Panteleeva, 1950; Lloyd, 1951; Lloyd and Parry, 1954), a reaction which is said not to occur in leucocytes (Kellie and Zilva, 1935). Barkhan and Howard (1958) found that all the vitamin C within the leucocyte is in the reduced condition, but this is difficult to reconcile with the AA-oxidising action of these cells as

claimed by Kellie and Zilva (1935), and by Lloyd (1951). In contrast to Lloyd's earlier conclusion, Lloyd and Sinclair (1953) later found that the TAA content of the white cell could be increased if DHA was added to a suspension of such cells, but not if AA was added.

The investigation of this problem of the metabolism of vitamin C by the leucocyte therefore formed the second subject of this work.

Summary

The objects of this investigation were twofold:

1. Comparison of leucocyte concentrations of vitamin C before and after surgery. By this means it was hoped to clarify the problem of increased post-surgical requirements of the vitamin.
2. Investigation of vitamin C metabolism in the leucocyte.

Materials and Methods

Reagents for Determination of AA

2:6 - Dichlorophenolindophenol.

A stock solution (1 mg./ml.) of the sodium salt was made up at weekly intervals and kept at 5°. A working solution of 4 mg./100 ml. was prepared from this stock immediately before each series of analyses.

Metaphosphoric acid

Approximately 3 gm. portions of metaphosphoric acid stick were weighed out and kept in tightly stoppered tubes. As required, the appropriate volume of distilled water was added to give a 3% (w/v) solution, which was kept in the refrigerator when not in use. This was prepared daily.

Trisodium citrate 12% aqueous solution (w/v).

CMB (L. Light & Co. Ltd.) 0.2% (w/v) in 0.05N NaOH.

Reagents for Determination of TAA

Trichloroacetic acid 6% aqueous solution (w/v).

2:4-Dinitrophenyl- 2% solution (w/v) in 9N H_2SO_4 .
hydrazine

Activated charcoal "Norit NK" (Hopkins & Williams
Ltd.)

85% Sulphuric acid Add 9 volumes of AnalaR
 H_2SO_4 (S.G. 1.84) slowly to
1 volume distilled water in
a beaker surrounded by ice.

Thiourea 10% (w/v) in 50% (v/v)
aqueous ethanol.

Reagents for Determination of GSH

Phosphate buffers (pH 6.8) were prepared according to Datta and Grybowski (1961), at $I = 0.2$ (strong) and $I = 0.05$ (weak).

Di-(5-carboxy-4-nitro-phenyl) disulphide (Aldrich Co., Wis., U.S.A.)

10 mg. of this compound were dissolved in 25 ml. of "strong" buffer, giving a 40 mg./100 ml. (mM solution).

Sodium Hydroxide (AnalaR) 0.25 N aqueous solution.

AA (BDH) An aqueous solution containing 1 mg./ml. was prepared immediately prior to use. From this solution standards covering the range 0 - 20 $\mu\text{g./ml.}$ were also prepared and used for calibration purposes.

DHA was prepared just before use by oxidation of AA with p-benzoquinone (Patterson, 1950). This preparation yields a solution containing approximately 100 mg. DHA/ml., appropriate dilutions to facilitate delivery of required amounts being made as necessary.

GSH (L. Light & Co., Colnbrook, Bucks.) An aqueous solution containing 1 mg./ml. was prepared and standards of 0 - 80 $\mu\text{g./ml.}$ used for calibration purposes.

Isolation of leucocytes

Siliconised glassware was used throughout in the preparation of leucocyte suspensions. Approximately 20 ml. blood, or a smaller volume if leukaemic blood was being

used, was collected in the morning from subjects who had fasted since the previous evening. It was delivered into a universal container to which had been added, as anti-coagulant, 0.4 ml. of 10% (w/v) ethylene diamine-tetracetic acid (di-potassium salt). After mixing, the blood was chilled in ice for ten minutes. It was then centrifuged slowly (10 - 30xg.) for ten minutes, and the leucocyte-platelet rich supernatant plasma was removed. When dealing with normal blood, phytohaemagglutinin preparations (Wellcome brand) were sometimes used, to accelerate erythrocyte sedimentation, but with leukaemic blood this was found to be unnecessary.

From this point in the procedure, two methods of isolation were used. The choice of method depended on the problem being investigated:-

- (1) When the estimation of TAA and AA leucocyte levels was the principal object, the presence of platelets would not influence the result, since they were being counted and a correction applied as described later.
- (2) For metabolic experiments leucocytes were prepared as free as possible from contaminating platelets, since it could not be assumed that the metabolism of vitamin C in these different types of cells would be similar.

The Preparation of leucocytes for TAA and AA determination.

A portion of the platelet and leucocyte-rich super-

natant plasma was removed for counting, and the remainder transferred to a tapered 10 ml. graduated centrifuge tube. After centrifuging at 1600xg. for ten minutes, the supernatant was carefully decanted from the white sediment and counted for leucocytes and platelets. The numbers of each class of cell in the sediment were calculated as shown in the following equation:-

$$C_1 - C_2 \times 10^3 \times \text{volume of initial suspension (ml.)}$$

In the above formula C_1 and C_2 are the first and second cell counts expressed as cells/c.mm.

Plasma was drained off for a few minutes, and the sediment of leucocytes and platelets was then resuspended in a volume of physiological saline to yield a cell population of $1.5 - 2.0 \times 10^4$ /c.mm. This suspension was then used in the analysis for TAA or AA, or both.

The preparation of leucocytes for metabolic investigations.

The platelet-leucocyte rich plasma was transferred to narrow tubes (0.5 x 5 cm.) and centrifuged at 450xg. for ten minutes. The supernatant was discarded, as well as the upper 0.5 cm. of the column of white sediment, which contained the majority of the platelets. Only the central 1 - 1½ cm. long column of leucocytes was used for these experiments, the small number of sedimented erythrocytes at the bottom of the tube also being discarded.

With normal blood, the leucocyte sediment was resuspended in a volume of saline equal to half that of the original plasma, and a portion removed for counting. With leukaemic blood the same procedure was used but a dilution was usually made to bring the white-cell count to within the limits of $1.5 - 2.0 \times 10^4$ cells/c.mm

Counting Technique

Leucocytes

Leucocyte counts were carried out on saline suspensions, using a Coulter-Electronic Counter (Model D) according to the method of Richar and Breakell (1959). 40 μ l. of cell suspension was added to 20 ml. of physiological saline, and counted after standing in contact with 100 μ l. of saponin for four minutes. Saponin was used to lyse any erythrocytes still present.

With blood from patients with leukaemia, visual examination of Leishmann-stained films was also carried out to establish the proportion of early and mature cells present. A comparison of the accuracy of the standard haemocytometer method and the method using the electronic instrument showed that the former procedure in skilled hands had a standard error of 20.5 per cent. and the latter a standard error of 7.5 per cent. (Richar and Breakell, 1959)

Platelets.

The platelets were counted visually using formol-

citrate diluent (Dacie, 1956). Several squares were counted for each specimen and the mean taken.

Preparation of Extracts.

Leucocyte suspensions, prepared as previously described, were deproteinised within an hour of the withdrawal of the blood, or at fixed time intervals during a metabolic experiment.

Meta-Phosphoric acid Extracts.

2 vols. of the leucocyte suspension in saline were added, with shaking, to 3 vols of 3% (w/v) meta-phosphoric acid. After standing for 5 minutes, precipitated protein was removed by centrifugation. This yielded a supernatant containing the vitamin C in 1.8% HPO_3 , in which experiment had shown that the vitamin was stable.

Trichloroacetic acid Extracts.

One volume of leucocyte suspension was added to 3 vols. of 6% (w/v) trichloroacetic acid, the mixture allowed to stand for 15 minutes, and then centrifuged.

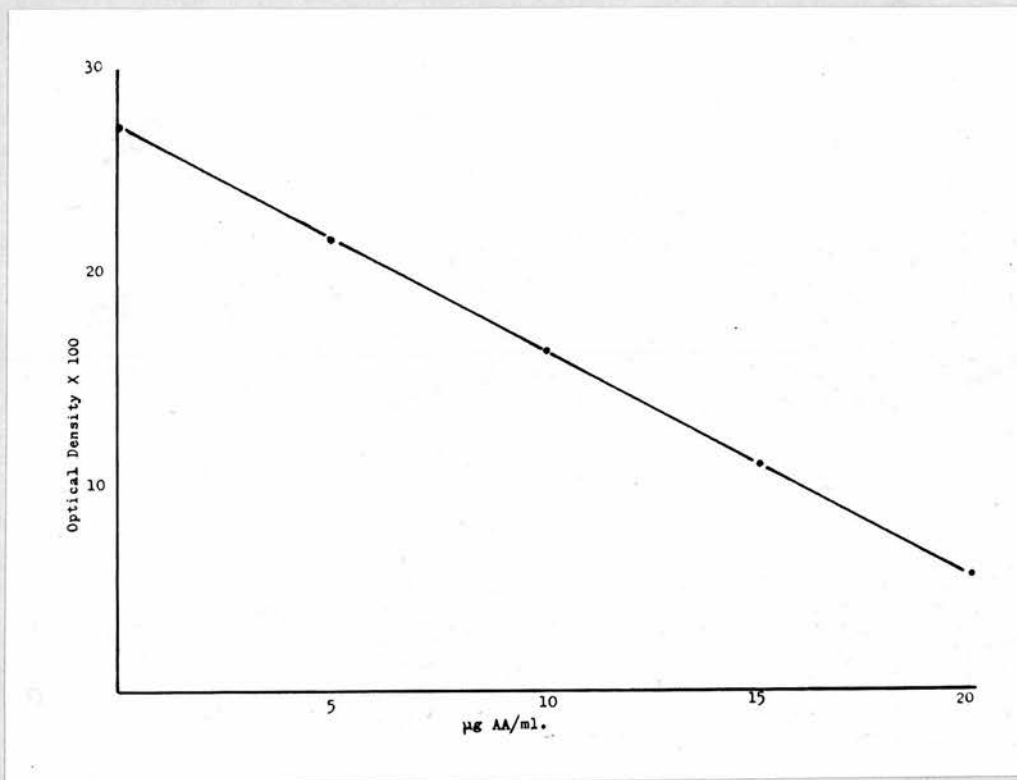
Analytical Methods.

AA Determination

AA was estimated by the indophenol method of Evelyn, Malloy and Rosen (1938), as modified by Owen and Iggo (1956). Use was also made of CMB as suggested by the latter authors, to suppress interference due to thiol compounds, since glutathione is known to occur in leucocyte preparations.

FIGURE 1

Calibration for AA estimation using the
modification of the dichlorophenol-indophenol
method of Owen and Iggo (1956).



(Contopoulos and Anderson, 1950; Hardin, Valentine and Lawrence, 1954).

Procedure:

To 3 volumes of the supernatant from the meta-phosphoric acid extract was added 1 volume of CMB reagent. After standing for 5 minutes the precipitate was removed by centrifugation. 2.0 ml. of the supernatant was transferred to a 10 mm. cuvette, followed by 0.5 ml. of 12% sodium citrate and 1.0 ml. of indophenol reagent. After rapid mixing the optical density of the solution was measured against water at 520 mμ, exactly 30 seconds after addition of the indophenol. Standard solutions of AA were processed in exactly the same manner. This resulted in "standardisation" of the indophenol reagent which was carried out daily. Figure 1 shows the calibration for this method.

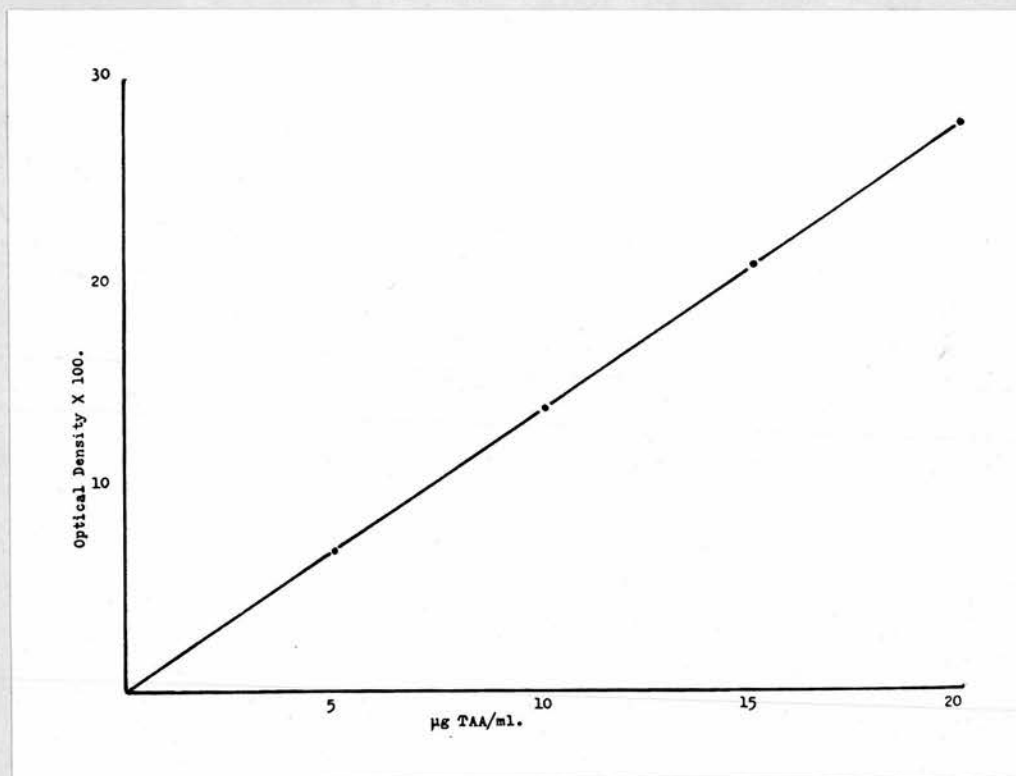
The reproducibility of the results was tested. The range when 8 determinations on the same leucocyte suspension were carried out was 3.5 - 3.7 μg./ml. The mean result was 3.6 ± 0.1 μg./ml.

TAA Determination.

TAA was estimated by the method of Roe and Kuether (1943). In this method trichloroacetic acid extracts are treated with activated charcoal as an oxidant, to convert AA into DHA, prior to formation of the coloured 2:4-dinitrophenylhydrazone derivative. Addition of 85% sulphuric acid

FIGURE 2

Calibration for TAA estimation by the
method of Roe and Kuether (1943).



to this derivative yielded a coloured solution, which occasionally required further dilution with the acid to bring the final concentration into the range of the standard curve.

Procedure:

0.5 gm. of Norit was added to the trichloroacetic acid extracts of the leucocyte suspensions and after shaking vigorously for several minutes were filtered. 4 ml. of the filtrates were transferred to tubes and 1 drop of thiourea reagent added, followed by 1 ml. of 2:4-dinitrophenylhydrazine. After mixing, the tubes were incubated for 3 hours in a 37° water bath. At the end of this time the tubes were removed to iced water and 4 ml. of 85% H_2SO_4 added drop by drop, mixed and left for 30 minutes. The optical density of each solution was then read at 540 mμ in 20 mm. cells against a blank made with 4.5% trichloroacetic acid instead of extract. Figure 2 shows the calibration for this method.

The reproducibility of the results was tested by carrying out 7 determinations on the same leucocyte suspension. The range was 3.6 - 4.0 μg/ml. The mean result was 3.8 ± 0.2 μg/ml.

Estimation of glutathione.

In certain experiments it was necessary to estimate the concentration of GSH in leucocytes. The method used was a modification (Jocelyn, 1962) of the original method of

Ellman (1959), who reported that the aromatic disulphide, di-(5-carboxy-4-nitro-phenyl) disulphide is reduced at a pH of 8.0 by non-protein SH to the corresponding aromatic thiol compound; this has an intense yellow colour which can be measured spectrophotometrically at 412 mμ. Some interference by protein SH, however, takes place. Jocelyn's modification (1962) depends on the fact that non-protein SH groups react with the disulphide at pH 6.8, with no interference from protein SH, whereas both protein and non-protein SH (total SH) can be estimated by bringing the reaction mixture to pH 7.6. Only the method for non-protein SH was used in this work.

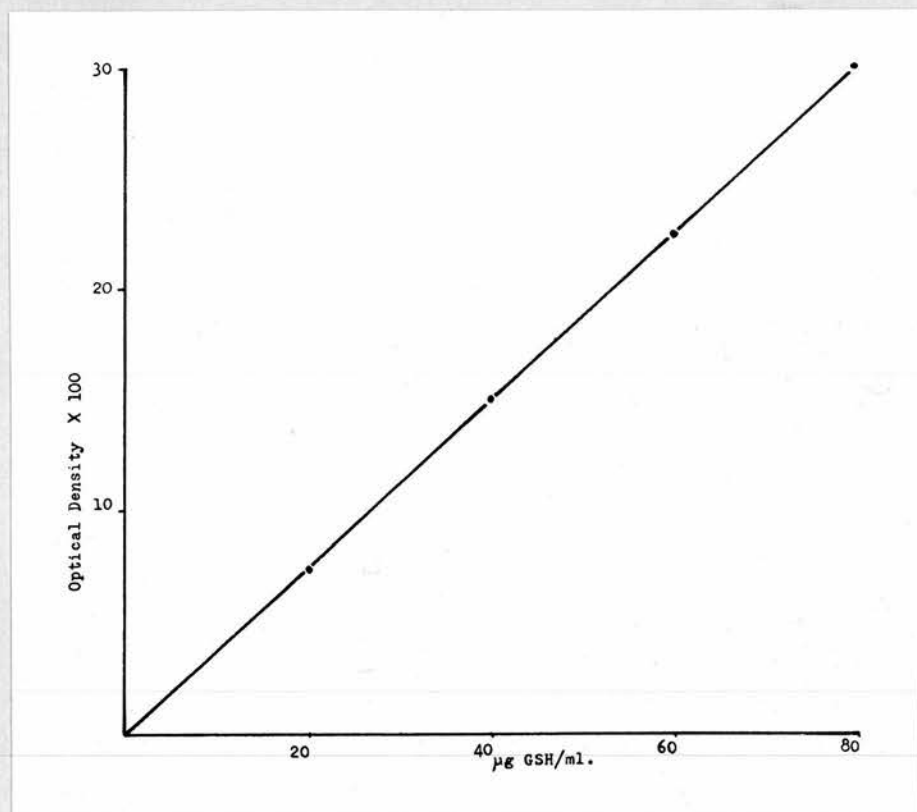
Method of GSH estimation.

Analyses of GSH in leucocytes were made on the supernatant after the protein precipitation stage in the AA estimation. After centrifugation, 3 ml. of supernatant was used for AA estimation and 1 ml. of the remainder was used for GSH assay in the following manner.

1 ml. of 1.8% metaphosphoric acid as a blank and 1 ml. of supernatant from the AA estimation were transferred to 3" x $\frac{1}{2}$ " test tubes. After neutralising with 0.9 ml. of 0.25N NaOH, 3.1 ml. of weak phosphate buffer (pH 6.8) was added to each tube, mixed well, and then 1 ml. of the colour reagent in strong buffer (pH 6.8) was added. The optical density of the test was read against the blank after five

FIGURE 3

Calibration for Glutathione Estimation
by the method of Jocelyn (1962).



minutes in the EEL Spectra, at 412 mμ in the 20 mm. cuvettes.

The calibration curve for this method is shown in Figure 3.

Spectrophotometer.

An EEL Spectra X 151 was used for all optical density measurements.

Expression of Results.

The leucocyte, representing as it does such a small percentage of the volume of whole blood, yields results of quantitative assays for which accurate expression is difficult to achieve. Early investigators such as Stephen and Hawley (1936) and Constantinides (1947), expressed their results on a wet weight basis only; Butler, Cushman and MacLachlan (1943) in terms of the volume of the buffy-cell layer; and Bessey, Lowry and Brock (1947), attempting greater accuracy, expressed their results per 100 gm. dry weight, after relating the AA content to the acid insoluble phosphate.

Barkhan and Howard (1958) and Denson and Bowers (1961) based their results on the number of individual cells determined by direct counting. The same procedure has been adopted in the present work and results have been expressed as μg AA or TAA/ 10^9 leucocytes.

Calculation of Results

Concentrations of TAA or AA in solution or in cell suspension are expressed as μg./ml. in the first instance.

Intracellular concentrations have been calculated by means of the following formula:-

$$\mu\text{g}/10^9 \text{ leucocytes} = U \times \frac{10^9}{\text{No. of leucocytes in 1 ml. suspension}}$$

where U is the concentration of TAA or AA in $\mu\text{g./ml.}$

Significance in Results.

The range of variation in the analytical procedures for AA and TAA has been shown to be 0.2 $\mu\text{g./ml.}$ and 0.4 $\mu\text{g./ml.}$ respectively. This means that for a leucocyte suspension with a count of 1.5×10^4 cells/c.mm., the above ranges of variation, when expressed as $\mu\text{g./}10^9$ cells are 13 $\mu\text{g.}$ and 26 $\mu\text{g./}10^9$ cells respectively. No significance can be given to differences in results which vary from each other by less than these amounts.

On the other hand, if the cell count on the suspension under examination was 0.5×10^4 cells/c.mm., the respective ranges of variation in leucocyte AA or TAA concentration would be 40 $\mu\text{g.}$ and 80 $\mu\text{g./}10^9$ cells. Significant differences in terms of $\mu\text{g./}10^9$ leucocytes therefore depend ultimately on numbers of leucocytes present.

To permit comparison of results, it was necessary to standardise the cell population analysed. This was the reason that cell suspensions were adjusted to contain $1.5 - 2.0 \times 10^4$ cells/c.mm. prior to analysis. Differences

in results of 10 μg . AA/ 10^9 cells and of 20 μg . TAA/ 10^9 cells were then known to be significant.

Correction for Platelets.

Barkhan and Howard (1958) determined the vitamin C content of leucocytes and platelets separately and found that both had similar AA concentrations. This confirmed the earlier work of Butler and Cushman (1940).

Preliminary calculations in the present studies revealed that, in the "buffy-layer" of normal human blood, the proportion of the total volume contributed by the platelets was almost as great as that contributed by the leucocytes.

The problem whether platelet contamination of the buffy layer of blood introduced a serious error in estimations of leucocyte vitamin C was assessed by calculating the percentages which platelets and leucocytes would be expected to contribute to their combined volumes.

The mean volume of a platelet was taken to be $16 \mu^3$ (Barkhan and Howard, 1958), and the average volume for a leucocyte was assumed to be $895 \mu^3$. The average volume for a leucocyte was computed from the volume of a granulocyte ($1046 \mu^3$) and of a lymphocyte ($594 \mu^3$) (Blitzen and van den Bergh, 1945), and assuming a proportion of 2 of the former cells to 1 of the latter in normal blood (Wintrobe, 1956).

Assuming platelet and leucocyte counts to be in the

middle of the normal ranges for each of these types of cells, the volume which each would occupy in 1 c.mm. of plasma was calculated as follows.

Normal range of platelet count is 200,000 - 500,000/c.mm.

Average count = 350,000/c.mm.

$$\begin{aligned}\text{Volume occupied by platelets} &= 350,000 \times 16 \mu^3 \\ &= 56 \times 10^5 \mu^3\end{aligned}$$

Normal range of leucocyte count is 5,000 - 10,000 cells/c.mm.

Average count = 7,500 cells/c.mm.

$$\begin{aligned}\text{Volume occupied by leucocytes} &= 7,500 \times 894 \mu^3 \\ &= 67 \times 10^5 \mu^3\end{aligned}$$

Calculating the percentage contribution of each to the total volume.

% Volume due to Platelets

$$\begin{aligned}&= \frac{56}{123} \times 100 \\ &= 46\end{aligned}$$

% Volume due to Leucocytes

$$\begin{aligned}&= \frac{67}{123} \times 100 \\ &= 54\end{aligned}$$

In terms of this calculation, leucocytes and platelets contributed comparable proportions of the vitamin C of the buffy layer, and it was decided to enumerate both of these cells in specimens and to correct for the platelets as outlined below.

Since the presence of normal numbers of platelets would seriously affect the accuracy of leucocyte AA determinations, the volume of the platelets was calculated and their

effect allowed for in the following way.

Average volume of a leucocyte = $895 \mu^3$ (Range $869 - 921 \mu^3$)
(Blitzen and van den Berghe,
1945; Wintrobe, 1956)

Average volume of a platelet = $16 \mu^3$ (Range $13.9 - 18.1 \mu^3$)
(Barkhan and Howard, 1958)

The ratio of the volume of the leucocyte to that of the platelet is therefore approximately 56, calculating from the average volumes of these cells, and the range of this ratio is $48 - 64$ (56 ± 8) calculating from the extremes of the volume ranges as indicated by the authors concerned. Hence, if the number of platelets is divided by a factor of 56, the result approximates to the number of leucocytes to which the original number of platelets would be equivalent. For example, using the same average counts as in the previous calculation:-

Platelet count = 350,000/c.mm. Leucocyte count = 7,500 cells/cmm.

$$\approx \frac{350,000}{56} \quad \text{leucocytes}$$

$$\approx 6,250 \quad \text{leucocytes}$$

"Equivalent" leucocytes present in sample

$$= 7,500 + 6,250 \text{ cells/c.mm.}$$

$$= 13,750 \text{ cells/c.mm.}$$

Use of the extreme values for the conversion factor would result in the range of the final "equivalent" cell count

being 13,000 - 14,800 cells/c.mm. which represents a deviation of $\pm 6\%$ from the originally assessed count of 13,750 cells/c.mm.

Interconversion of alternative expressions of concentration.

In the body of this thesis when leucocyte concentrations are referred to, with reference to the work of others, they will be given both in $\mu\text{g}/10^9$ cells and in mg./100 ml. cells. The one which was estimated originally will be given first, and the computed one in brackets after it. These values have been interconverted by a method given by Barkhan and Howard (1958). Taking the average volume of the leucocyte to be $895 \mu^3$, and by assuming the specific gravity of the cells to be unity, it follows that 10^{12} cells occupy a volume of 895 ml.

Hence the results originally calculated as $\mu\text{g}/10^9$ cells can also be expressed as mg./ 10^{12} cells

or as mg./895 ml.

The results in mg./100 ml. are therefore $\frac{\text{mg.}/10^{12} \text{ cells}}{8.95}$

RESULTS

Part I

Studies on Leucocytic Vitamin C

Studies on Leucocytic Vitamin C

Introduction

In a review of the biochemistry of vitamin C Lloyd and Sinclair (1953) stated that although the high concentration of leucocytic vitamin C had been known more than fifteen years previously (Stephen and Hawley, 1936; Cuttle, 1938) surprisingly little investigation of the dynamics of the vitamin in its relation to the leucocyte had been undertaken. This statement was equally true when the present studies commenced.

Initial investigations

The initial experiments were concerned with the following factors which might be important in subsequent work using leucocyte suspensions.

1. The stability of leucocytic vitamin C during the period that the cells were in suspension in physiological saline.
2. The efficiency of the process of extraction of vitamin C from intact leucocytes.
3. Recoveries of added AA.

During investigations of these factors, leucocyte suspensions in saline as prepared for metabolic investigations were used. The results of this initial work will now be presented.

1. Stability of leucocyte vitamin C

Single aliquots of a saline suspension of leucocytes

TABLE 1

Stability of Vitamin C in leucocytes suspended
in physiological salineExperiment 1. (cell count 15,000/c.mm.)

Time in contact with saline before acid precipitation (hrs.)	TAA			AA		
	µg./ml. of sus- pension	µg./10 ⁹ cells	% of initial concen- tration	µg./ml. of sus- pension	µg./10 ⁹ cells	% of initial concen- tration
0 (control)	3.4	227	-	3.3	220	-
$\frac{1}{2}$	3.3	220	97	3.3	220	100
1	3.4	227	100	3.3	220	100
3	3.2	214	94	3.0	200	91
5	3.1	207	91	2.8	187	85

Experiment 2. (cell count 16,400/c.mm.)

Time in contact with saline before acid precipitation (hrs.)	TAA			AA		
	µg./ml. of sus- pension	µg./10 ⁹ cells	% of initial concen- tration	µg./ml. of sus- pension	µg./10 ⁹ cells	% of initial concen- tration
0 (control)	4.1	250	-	4.1	250	-
$\frac{1}{2}$	4.0	244	97	4.1	250	100
1	4.1	250	100	4.1	250	100
3	3.4	208	83	3.4	208	83
5	3.0	183	73	3.1	187	75

were removed at the time intervals shown in Table 1 (Experiment 1), and added to the appropriate acid precipitant. By reference to the control, which was deproteinised immediately, it could be shown that no loss of TAA or AA occurred at room temperature if deproteinisation was carried out within an hour. If longer periods of time were allowed to elapse before deproteinisation, some loss in AA did occur, which was outside the limits of experimental error.

The decrease in AA could have occurred either intracellularly or extracellularly; a second experiment on the stability of leucocytic vitamin C was carried out to clarify this problem.

A saline suspension of leucocytes was prepared as before. In the present study, however, aliquots of cell suspensions removed for analysis were not added to the acid precipitant in the saline in which they had remained suspended, but were centrifuged and resuspended in fresh saline prior to analysis. No significant loss of cells occurred during centrifugation. These results are shown in Table 1 (Experiment 2). After 30 mins. and 1 hr. similar concentrations were found as in the control, corresponding with the results obtained in the previous study and indicating that the vitamin was still within the cell after suspension in saline for 1 hr. However, after 3 hrs. and 5 hrs. a smaller percentage of the initial TAA and AA was found remaining in

TABLE 2

The efficiency of extraction of leucocyte
vitamin C

<u>Experi-</u> <u>ment</u>	<u>Leucocyte</u> <u>Preparation</u>	<u>TAA ug./ml.</u>	<u>AA ug./ml.</u>
1	Intact cells 1	2.25	2.25
	Lysed cells 1	2.40	2.30
2	Intact cells 2	3.10	3.20
	Lysed cells 2	3.25	3.15
3	Intact cells 3	3.95	3.80
	Lysed cells 3	3.80	3.85

Each of the above results is the mean of duplicate analyses.
As stated in the Experimental Section the accuracy of the TAA
determination is $\pm 0.2 \mu\text{g./ml.}$ and of the AA determination the
accuracy is $\pm 0.1 \mu\text{g./ml.}$

the cells than had been found in the corresponding whole suspensions in the previous experiment.

These results indicate that,

- (1) after one hour the diffusion of vitamin C from the leucocyte is detectable, and
- (2) the decrease of TAA and AA shown to occur is brought about by extracellular oxidation.

2. Extraction efficiency.

It was shown that addition of leucocyte suspensions to trichloroacetic or metaphosphoric acids resulted in protein precipitation, and extracted all the vitamin C from within the white cells. Several duplicate analyses were carried out using intact and lysed cells. Lysis was achieved by freezing and thawing the cells alternately, about six times. The efficiency of this process was confirmed by counting immediately prior to analysis.

The results are shown in Table 2, where it can be seen that the extraction of vitamin C from intact cells was as efficient by the methods of acid precipitation employed as by lysis of the cell.

3. Recovery of added AA.

Only one recovery experiment was carried out. The appropriate volume of the AA solution was added to the cell suspension to yield the required concentration and the preparation was immediately deproteinised. The recoveries

TABLE 3.

Recovery of added AA.

<u>µg./ml. added to leucocyte preparation.</u>	<u>TAA µg./ml.</u>	<u>% recovered.</u>	<u>AA µg./ml.</u>	<u>% recovered.</u>
nil	1.40	-	1.40	-
1.5	2.95	102	2.90	100
2.5	3.85	99	3.95	101
3.0	4.50	102	4.40	100

Only one experiment of this kind was carried out and each of the above figures is the mean of duplicate analyses.

obtained were satisfactory. Determinations were made in duplicate and the concentrations shown are the mean results (Table 3).

Conclusions.

(1) Loss of vitamin C from the leucocyte (Table 1) begins to occur by diffusion and subsequent oxidation after one hour of exposure to physiological saline at room temperature.

On the basis of these results it was considered important to reach the stage of protein precipitation with as little delay as possible.

(2) In the course of these experiments, all the vitamin C within the leucocyte has been found to be in the reduced condition; the need to estimate DHA or DGA in the surgical experiments to be described later therefore did not arise. In the following metabolic experiments where DHA was prepared from AA and added to leucocyte suspensions, the concentration of DHA was found by subtracting the AA value from that found for TAA, since DGA was known not to be present initially.

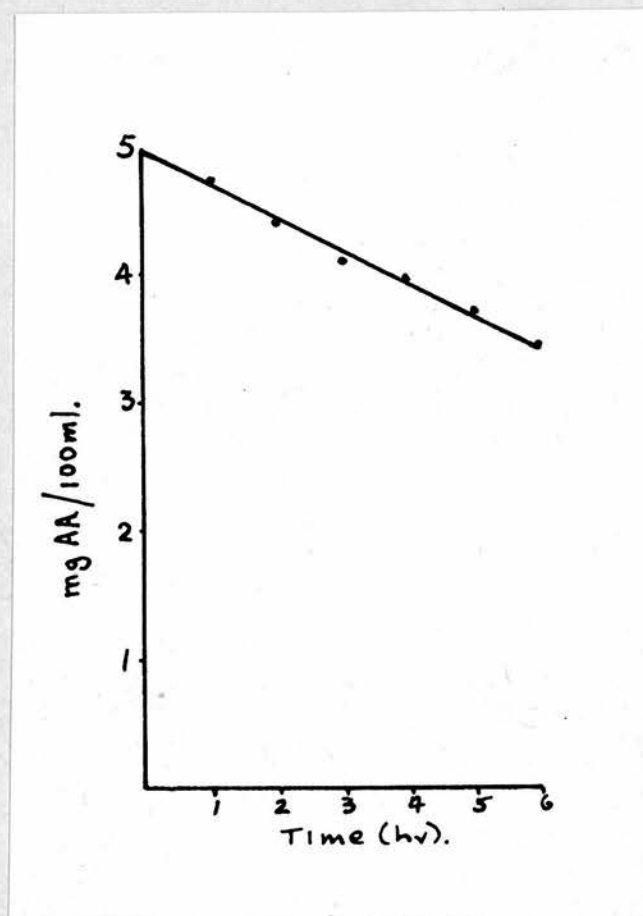
Leucocyte permeability to AA and DHA.

Introduction

It was planned to expose leucocyte suspensions in saline to known concentrations of DHA and AA for various periods of time, after which the cells would be centrifuged down, resuspended in fresh saline, and their TAA and AA

FIGURE 4.

The rate of Oxidation of AA
in Saline at 20°C.



contents determined. By this means it was hoped to discover if the leucocyte membrane was selectively permeable to one form of vitamin C only.

The rapidity of AA oxidation in aqueous solution at room temperature was a major problem in this investigation, since a leucocyte suspension exposed initially to only AA would come into contact with increasing amounts of DHA, while the AA content would be simultaneously decreasing. If the vitamin C content of leucocytes was found to increase under these circumstances, it would be impossible to determine to which form of the vitamin the leucocyte was permeable or if it was, in fact, permeable to both forms.

The rate of oxidation of AA in saline was investigated by making a 5mg% solution and estimating the concentration of the reduced vitamin periodically for six hours. The results are shown in Figure 4.

In the light of these results it was decided that experiments on leucocyte permeability to AA would yield reliable results only if limited to a period of 30 minutes.

Results.

Leucocyte suspensions in saline were exposed to a concentration of 50 $\mu\text{g.}/\text{ml.}$ of either AA or DHA at room temperature for intervals up to a maximum of 30 mins. and, after lapse of the appropriate time, the cells were centrifuged down, resuspended in the same volume of fresh saline,

and analysed for AA and TAA. Three experiments following this procedure were carried out. Similar results were obtained in each case. The results of one of these experiments are shown in Table 4. (see Appendix II)

Discussion

It was shown that within the time period studied no uptake of AA by the leucocyte could be demonstrated, in contrast to a rapid uptake and subsequent reduction of DHA. When the increase in leucocyte AA in such experiments was plotted against time, it was shown that the maximum concentration of AA in these cells had not been reached in the course of the experiments. The determination of a maximum value for leucocyte AA concentration was considered of interest, and it was decided to investigate how long the absorption of DHA by leucocytes would continue.

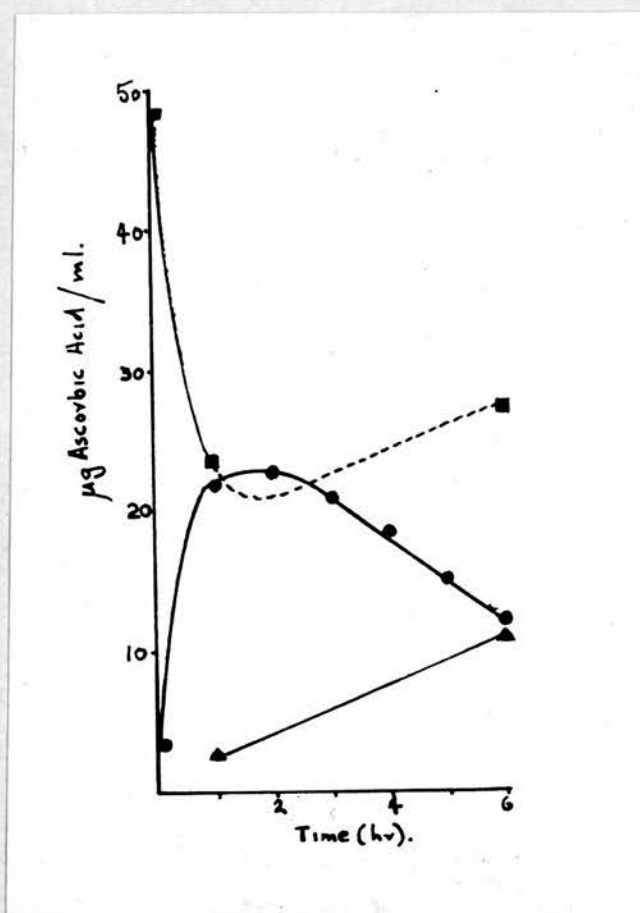
Behaviour of Leukaemic Granulocytes treated with DHA.

Introduction

To obtain a large enough leucocyte population for serial absorption studies, white blood cells from a case of acute myeloid leukaemia (untreated) were used. It should be emphasised that in the majority of experiments described in this thesis, mixed cell populations of leucocytes were used whereas, in the following two studies, 97% of the white cells present were of the myeloid series. The comparative biochemical metabolism of the myelocyte may vary

FIGURE 5.

Absorption and Reduction of DHA by leukaemic granulocytes in saline suspension.



The equivalent of 45 micrograms per ml. DHA was introduced into the suspension at time zero. The leucocyte count in this experiment was 4.7×10^4 cells per cmm. Other details are given in the text.

- leucocyte AA
- Supernatant TAA
- ▲ Supernatant AA

FIGURE 6.

Experimental procedure diagram
to Study 2.

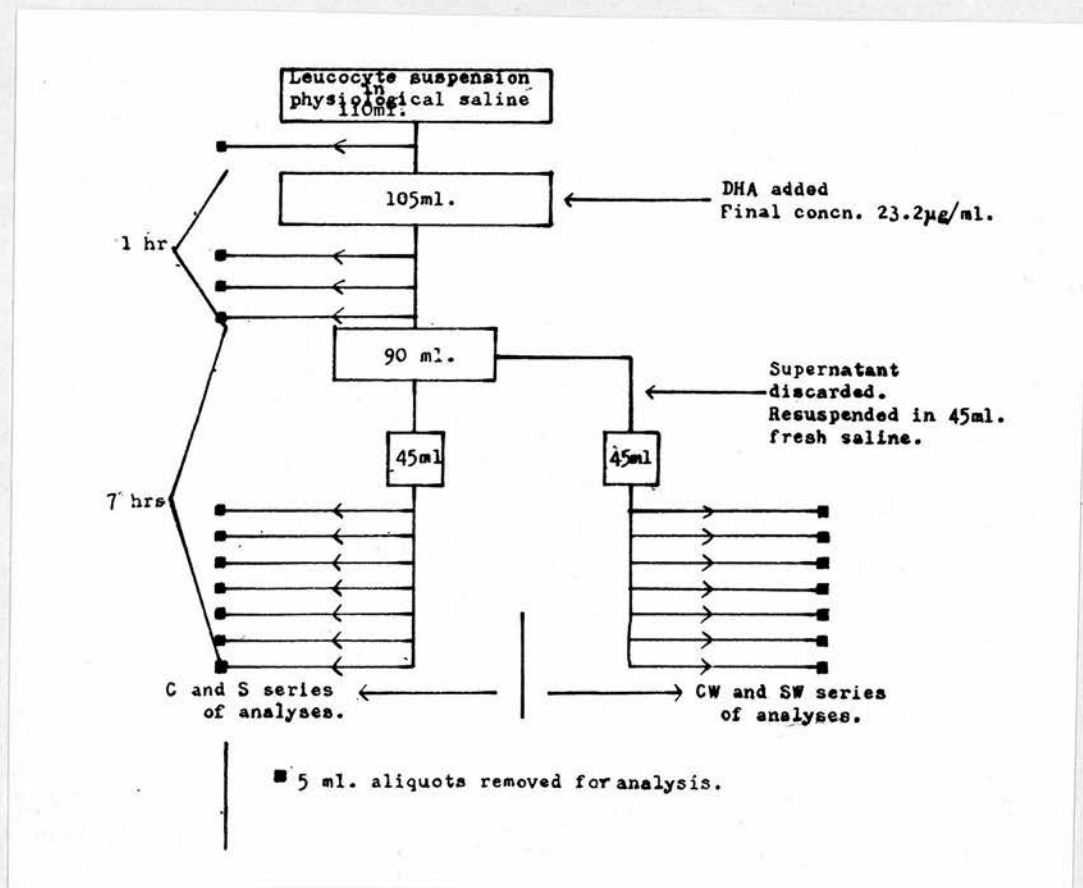
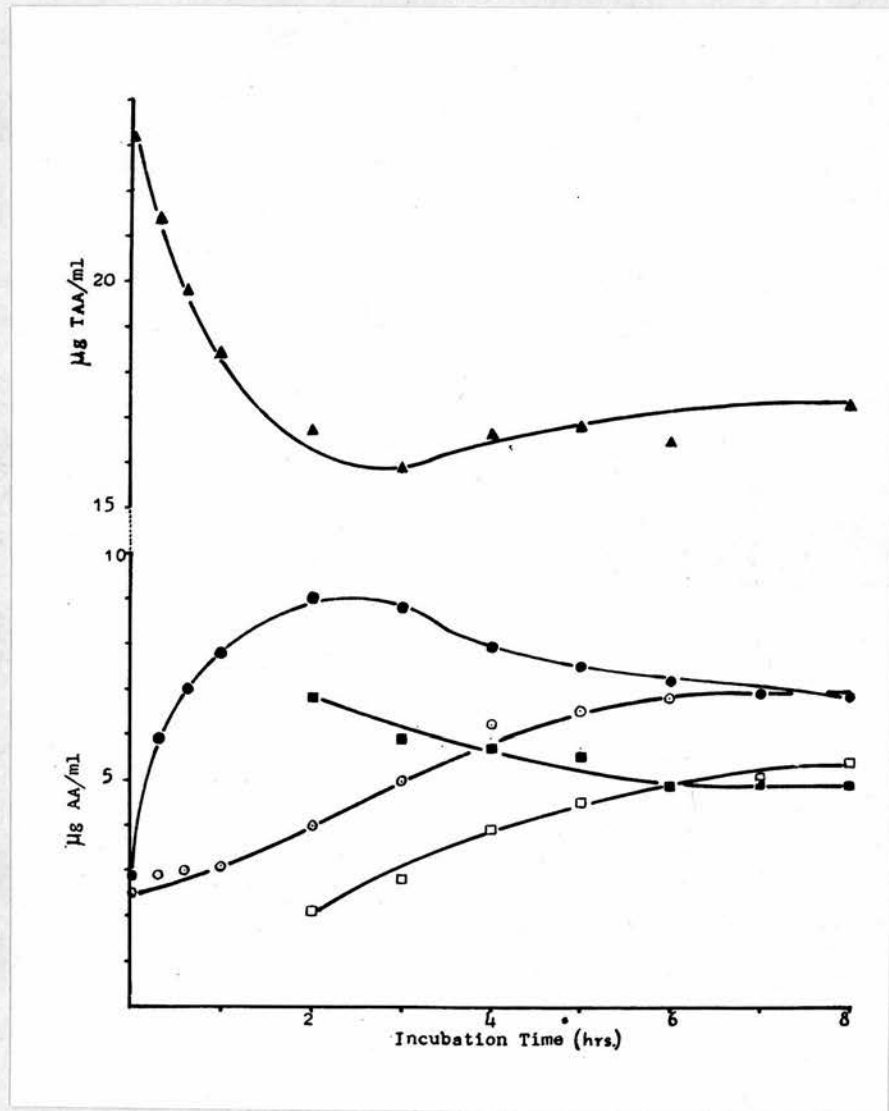


FIGURE 7.

**Absorption and Reduction of
DHA by leukaemic granulocytes**



Analyses on the original suspension
(S and C series of Figure 6)

- ▲ TAA concentration in saline supernatant.
- AA concentration in saline supernatant.
- Leucocyte AA.

Analyses on the resuspended leucocytes
(SW and CW series of Figure 6)

- AA concentration in saline supernatant.
- Leucocyte AA.

according to age. For this reason, differential counts were done to establish the proportion of early and mature cells present, with the object of being able possibly to relate results to known mixtures of cell types at a later date.

Experimental Study - 1.

Approximately 50 µg./ml. of DHA was added to a leucocyte suspension at 37°. At various intervals, aliquots were removed from the preparation and the cells analysed as before, after centrifugation and resuspension in fresh saline. The supernatant saline in contact with the cells during the incubation was also analysed for TAA and AA at the start, after one hour, and at the end of the experiment (Figure 5).

Experimental Study - 2.

To obtain a comprehensive picture of AA and DHA levels in a leucocyte suspension absorbing DHA, the previous experiment was repeated carrying out analyses for AA and TAA on the extracellular medium as well as on the cellular fraction at all stages of the incubation. Approximately 25 µg./ml. of DHA was added to the cell suspension instead of 50 µg./ml. as in the previous experiment.

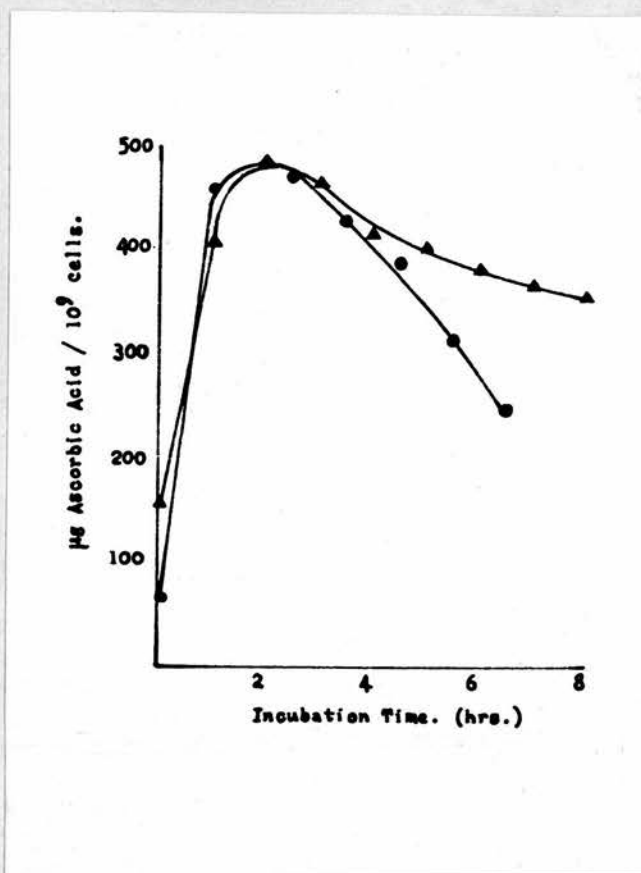
The experimental procedure was complex and relevant information is shown diagrammatically or stated (Figure 6). The white blood cells were obtained from the same leukaemic

subject as in the previous experiment. Absorption of DHA by the leucocytes was rapid and a corresponding fall in the TAA determination of the supernatant occurred (Figure 7).

Discussion

In Figures 5 and 7 concentrations of TAA or of AA have been expressed as $\mu\text{g./ml.}$ of cell suspension, or as $\mu\text{g./ml.}$ of supernatant saline, since in this form the changing distribution of the two forms of vitamin C within the system can be seen. If the endocellular AA in Figures 5 and 7 is expressed in terms of $\mu\text{g./10}^9$ cells and then graphed against time, we arrive at the results shown in Figure 8. In both experiments the maximum concentration of leucocyte AA reached was approximately $485 \mu\text{g./10}^9$ cells. After this maximum was attained the concentration began to fall, the rate of fall slowing considerably when a concentration of about $350 \mu\text{g./10}^9$ cells had been reached. Leukaemic cells are known to undergo rupture more easily than normal cells (Whitby and Britton, 1963) so that the appearance of AA in the extracellular environment could have been due to their disintegration. However, this was not the case in these experiments, as was shown by investigating cell stability over a 22 hr. period, under the same conditions (Figure 9). The rate of disintegration of these cells was not sufficient to account for the observed rate of increase of AA in the extracellular medium. The increasing concen-

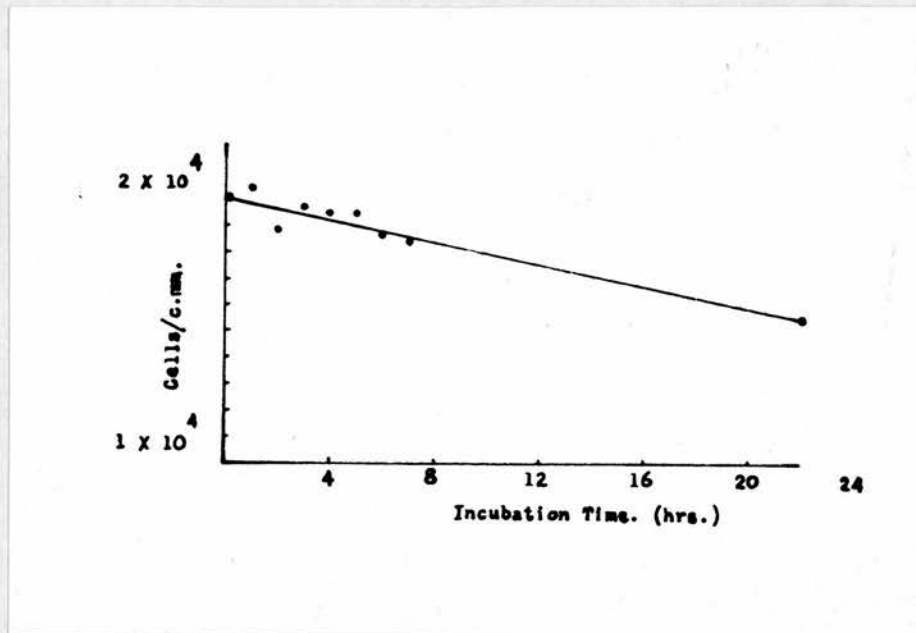
FIGURE 8.



Comparative rates of uptake by leukaemic granulocytes
exposed to different concentrations of DHA.

- cells exposed to 45 micrograms DHA/ml. (cell count 4.7×10^4)
- ▲ cells exposed to 23.2 micrograms DHA/ml. (cell count 1.87×10^4)

FIGURE 9.



Rate of disintegration of leukaemic granulocytes in
saline at 37°C .

The rate of destruction of these cells is only one quarter of that which would have to occur if the appearance of AA in the medium was due to leucocyte disintegration.

tration of AA must therefore be due to diffusion from leucocytes.

DHA - Reducing activity of leucocyte membranes.

Even when the leucocyte was absorbing DHA rapidly, in the two experimental studies just described, leucocytic TAA was always equal to the AA figure. This suggested that the absorption of DHA was in some way linked to its simultaneous reduction. A possible site of this rapid reduction was the cell membrane, and this hypothesis was tested.

Leucocyte membranes were prepared by the procedure which Kidd (1949) used for the isolation of the stroma of erythrocytes. The isolated membranes were resuspended in saline and exposed to DHA solutions containing 50 μ g./ml. for 30 min. TAA and AA determinations were then carried out. No reducing action could be observed in these studies on isolated membranes.

The absorption of DHA by the leucocyte must take place via the cell membrane, where the oxidised vitamin might become fixed temporarily. The present results do not preclude such a fixation of DHA by membranes but they indicate that the reduction of DHA previously observed requires the participation of cellular processes other than those associated with structural components of the membrane.

DHA - Reducing activity of lysed leucocyte preparations.

When Thomson et al (1956) investigated the reduction

of DHA by human erythrocytes, they found that either intact cells or haemolysed preparations could effect the reduction. They concluded that the site of reduction was within the erythrocyte and not on the wall of the cell. In view of the failure of leucocyte membranes to reduce DHA, shown in the previous section, a site within the leucocyte for this reduction was investigated by using the same technique as Thomson et al (1950) had used for erythrocytes.

A leucocyte suspension was lysed by alternate freezing and thawing. After complete lysis had been confirmed by counting, DHA (50 $\mu\text{g./ml.}$) was added to the preparation. After 30 min. the system was analysed for TAA and AA. No reduction of DHA had occurred. Addition of glutathione (100 $\mu\text{g./ml.}$) did not restore the capacity of DHA-reduction to these lysed cells.

A satisfactory explanation of the failure to detect any reduction of DHA in these studies requires further experimental work, but dilution of essential enzyme systems seems a possible reason.

The nature of the Leucocyte-reducing Mechanism

The effect of substances reported to inhibit the reduction of DHA by reacting with GSH was investigated. Schultze, Stotz and King (1938) used iodoacetate, and Thomson et al (1956) used p-chloromercuribenzoate (CMB) to suppress GSH reduction of DHA in haemolysed erythrocyte

TABLE 5

Uptake of DHA by leucocytes in the
presence of SH-blocking compounds

Systems in Saline Suspension	$\mu\text{g./ml.}$ of cell suspension	
	AA	TAA
Control Leucocytes	3.0	3.1
Leucocytes + DHA	11.3	11.3
Leucocytes + GMB + DHA	3.4	5.4
Leucocytes + Iodo- acetate + DHA	4.4	6.0

The concentration of DHA in the cell suspensions was
10 $\mu\text{g./ml.}$

Leucocytes were allowed to remain in contact with DHA
and SH-blocking compounds for 30 mins. before separation
and analysis.

preparations.

Iodoacetate and CMB were both employed in these investigations into the mechanism of leucocytic DHA reduction; these compounds were prepared at a concentration of 200 mg./100 ml., the former in saline and the latter in 0.05 N sodium hydroxide. It was necessary to neutralise the alkaline CMB solution prior to addition to the test suspensions. If neutralisation was not carried out, AA in the preparation was destroyed, due to the high pH of the alkaline solution.

In the first experiment, addition of the equivalent of 10 µg. DHA/ml. was made to two of three 3 ml. suspensions of leucocytes from the same population after having first added 1 ml. of iodoacetate solution and 1 ml. CMB suspension respectively to the two test suspensions. Ten minutes later the cells were centrifuged, resuspended in 3 ml. saline, and analysed for TAA and AA in the normal manner. Both iodoacetate and CMB had prevented the entry of any DHA into the leucocytes. This was shown by similar concentrations of TAA and AA being found in all tubes.

In a subsequent experiment (Table 5) in which the cells were allowed to remain in contact with DHA for a longer period (30 mins.) evidence was obtained to show that there was movement of DHA into the cell with very slight reduction in the presence of CMB, but with almost 50%

TABLE 6

GSH Concentration of Leucocytes in Leukaemia

<u>Subject</u>	<u>Condition</u>	<u>Total GSH</u> <u>mg/10¹⁰ cells</u>
O.H.	Normal	6.0
P.H.	Normal	5.8
M.M.	Chronic myeloid leukaemia	1.81
A.C.	Acute myeloid leukaemia	1.11
A.M ^C C.	Chronic myeloid leukaemia	2.51
A.G.	Acute myeloid leukaemia (under treatment)	2.81

Normal range of Hardin, Valentine and Lawrence (1954) 3.3 - 6.3

reduction in the presence of iodoacetate. This means that the passage of DHA across the leucocyte membrane and its commonly observed reduction are independent processes.

Levels of Glutathione in Leukaemic Myelocytes

Several determinations of leucocyte AA were carried out on cases of acute and chronic myeloid leukaemia, the results of which were consistently low, and have been reported in the second part of this thesis. On the other hand, it has been reported that leucocyte GSH levels are high in the leukaemias (Contopoulos and Anderson, 1950). It seemed worthwhile to investigate the GSH content of the white blood cells of leukaemic patients in view of the experimental finding reported earlier (Table 5) that SH groups are involved in the reduction of DHA. The results of these investigations are shown in Table 6 where it can be seen that the GSH content of leukaemic leucocytes was not raised.

Discussion

Vitamin C Metabolism in the Leucocyte

When Kellie and Zilva (1935) reported that leucocytes failed to reduce DHA, their evidence was indirect, being based on the inability of these cells to protect AA from spontaneous oxidation in solution; homogenates of other tissues act in this way. Their experiments were a measure of the inhibitory effect of the tissue concerned on the oxidation of AA in aqueous solution rather than an indica-



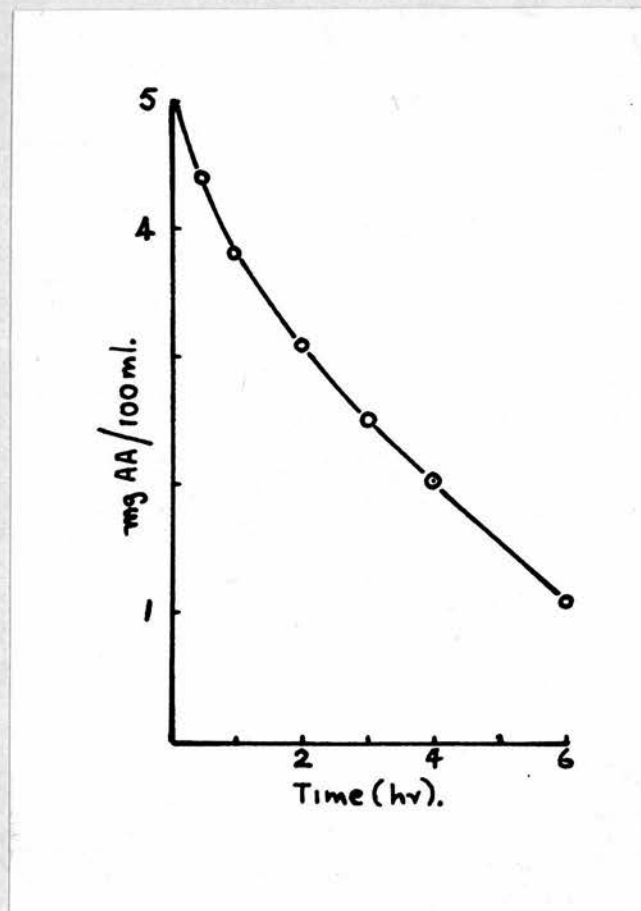
tion of any reducing capacity towards DHA.

Heinemann (1941) observed that AA added to whole blood was taken up by the leucocytes, but he failed to take account of the chemical changes which this form of the vitamin undergoes when added to blood or plasma. The metabolic activities of AA and DHA in relation to the leucocyte, which have been presented in this part of the thesis, are borne out by the findings of Denson and Richards (1962) and of Denson and Bowers (1961), but do not correspond with their conclusions.

Firstly, Denson and Richards (1962) determined plasma and leucocyte levels of TAA before, and three hours after, an oral dose of 700 mg. of AA in several subjects. Secondly, they allowed the leucocytes from these subjects to remain in contact with the plasma of the blood from which they were derived for a further four hours. In the first part of their investigation, typical findings were that the plasma level increased from 0.7 mg./100 ml. before to 2.0 mg./100 ml. after the AA administration, while the corresponding leucocyte levels were virtually stable at $260 \mu\text{g.}/10^9$ cells and $270 \mu\text{g.}/10^9$ cells. However, the same cells, after standing in contact with the parent serum for 4 hours at 4° , gave values of $300 \mu\text{g.}/10^9$ cells and $520 \mu\text{g.}/10^9$ respectively. They interpreted these findings as meaning that leucocytes can absorb AA, but do not comment on their in-

FIGURE 10.

The Rate of Oxidation of AA in plasma at 4°C .



ability to demonstrate any increase in leucocyte TAA in vivo three hours after the ingestion of 700 mg. of AA.

On the basis of the results in the first part of this thesis, the interpretation of the results of Denson et al (1961, 1962) would be that the leucocytes, in vivo, failed to take up vitamin C because the vitamin was in the reduced form in the blood stream. Only a small amount of DHA is normally present in blood (Linkswiler, 1958; Stewart, Horne and Robson, 1953), and this compound would be expected from the author's results to be the source from which the vitamin C of the leucocyte is derived. The increase in vivo would be expected to be very slow, since erythrocytes are competing with leucocytes for this compound (Panteleeva, 1950; Thomson, Iggo, Brownie and Stewart, 1956). The slow rate of increase of leucocyte AA in vivo has been confirmed by the work of Crandon et al (1961), on vitamin C-depleted pre-surgical patients, oral therapy having to be continued for weeks before the white cell level of the vitamin rose appreciably.

As has been shown (Figure 10), AA added to freshly separated plasma which is chilled and left at 4°C does not remain in the reduced condition. After four hours approximately 60% has been oxidised to DHA in which form it would then be available for reduction by leucocytes in experiments such as those of Denson and his coworkers.

The lack of any evidence of AA absorption, demonstrated in the experiments described here, obviates the need to postulate an active process as being responsible for the high concentrations of the vitamin found in leucocytes. Intracellular reduction maintains the DHA concentration gradient between the cell and its environment at the highest possible level and the passive diffusion of DHA across the leucocyte membrane satisfactorily explains the observed facts.

Factors Influencing the Diffusion of AA from the Cell.

The remarkable ability of the leucocyte to maintain a high internal concentration of AA has been confirmed in preliminary experiments (Table 1), and these results agree with observations on the failure of leucocytic vitamin C to diffuse into isotonic potassium oxalate (Bessey *et al.*, 1947), or into tungstic acid solution (Butler and Cushman, 1941).

The maintenance of high concentrations of AA by the leucocytes (20 - 40 mg./100 ml.), suggests that there may be some type of binding of the vitamin within these cells. Such binding, if it exists, is of a loose nature for it has been shown in the course of this work that AA liberated from lysed cells is as easily oxidised as AA in an aqueous solution. Furthermore, it has been shown that a slow release of AA from the leucocyte takes place (Figure 7).

Some interesting observations can be made from

Figures 5 and 7 regarding the factors controlling the diffusion of AA from the leucocyte to the plasma. There are two factors which appear to be involved.

If the curve for the appearance of AA in the extracellular medium of washed cells (Figure 7) is extrapolated backwards and parallel to the same curve for the supernatant of the cells which remained in contact with DHA, it can be shown almost to meet the origin. This suggests that AA began to diffuse from the cells when DHA was added to the medium. In support of this hypothesis, the results shown in Table 1 indicate that, from leucocytes suspended in saline, in the absence of DHA no diffusion of AA could be demonstrated over a period of 1 hour. Unfortunately, only TAA and AA estimations were carried out in the course of these experiments. Since TAA includes AA, DHA and DGA, the concentration of DHA remaining in the medium after $1\frac{1}{2}$ hours, when the increase in leucocyte AA ceases, is not known. Some idea of the quantity of DHA remaining may be surmised from the studies of Thomson et al (1956). Under similar experimental conditions, using erythrocyte suspensions, they showed that about 10% of DHA added to the medium remained after one hour, confirming the half-life measurements of this compound published by Kinsey (1950), who found it to be 20 minutes. It is reasonable to assume, therefore, that less than 5% of the initial supplement of DHA in the present experiments

remained after $1\frac{1}{2}$ hours.

The remarkable fact emerging from this study (Figure 7) is that diffusion of AA from leucocytes containing $154 \mu\text{g. AA}/10^9$ cells (Figure 8) was comparatively rapid at the beginning of the experiment when DHA was present; after 8 hours however, when DHA can reasonably be expected to have been oxidised to DGA, the rate of diffusion of AA from the cells was much slower, in spite of the concentration of AA within the leucocytes having risen to $354 \mu\text{g.}/10^9$ cells. A controlling factor in the rate at which AA diffuses from white blood cells may therefore be the availability of DHA in the extracellular medium.

The internal concentration of AA is the second factor which may influence the diffusion of AA from the leucocyte, since the rate at which this occurred was maximal during the second hour of incubation, (Figure 7) when the cellular concentration was at its peak and when the concentration of DHA in the surrounding medium was undoubtedly lower than its initial value. Also the appearance of AA in the extracellular fluid after 2 hours of (a) leucocytes isolated from the original medium and resuspended in fresh saline and (b) leucocytes remaining in contact with the original medium (Figure 7), was a mirror image of the decrease of AA in the respective leucocytes. This observation does not agree with Lloyd's (1951) suggestion that the leucocyte's internal concentration

of vitamin C might be controlled by the ratio of DHA to AA in the surrounding medium.

The rate of diffusion of leucocyte AA from cells in the absence of DHA could be shown to be logarithmic.

The Reducing Mechanism

Hopkins and Morgan (1936) were the first to show that GSH was necessary for the maintenance of the reduced condition of vitamin C within the tissues. It is interesting, therefore, to note the low values obtained for leucocyte GSH in the studies on leukaemic subjects (Table 6) in view of the correspondingly low values reported later for leucocyte AA obtained from subjects suffering from this condition. It is possible that the AA content of the leucocytes in vivo, in this case, is low because of the low GSH content being a rate-limiting factor in the reduction of DHA to AA by these cells. If both erythrocyte and leucocyte populations are competing for DHA, and leucocyte GSH is low, the final AA content of the leucocytes would also be expected to be low, as is indeed the case.

Failure to demonstrate membrane and lysed-cell DHA-reducing activity does not preclude the existence of such enzyme systems, since lack of activity in these preparations may have been due to dilution of essential factors. The fact remains that no DHA-reductase has as yet been demonstrated in animal tissues (Borsook, Davenport, Jeffreys and

Warner, 1937; Schultze et al., 1938), but the existence of a non-enzymic mechanism for DHA reduction has been claimed by Kinkawa (1944).

General.

An idea of the dynamic nature of vitamin C metabolism can be obtained from the fact that DHA, shown by many authors to be produced in vivo, (Martin, 1961; Salomon, 1957), can nevertheless not be shown to give rise to DGA (Damron, Monier and Roe, 1952; Iggo, Owen and Stewart, 1956; Miao, Kuo and Chen, 1957), although its half life period under physiological conditions has been reported as only 3 minutes (Ball, 1937).

Martin (1961) recently investigated the kinetics of distribution of the two forms of vitamin C, and emphasised that DHA is unionised and so more diffusible at the pH of the body than the negatively charged ascorbate ion. He showed that the volume of distribution of labelled AA approximated to the extracellular fluid volume, but that the volume of distribution of labelled DHA was much greater and must have included the intracellular volume also.

Summary of Results of Part I.

1. AA is not absorbed by human leucocytes. By contrast DHA is absorbed and then reduced to AA.
2. Only reduced vitamin C is found within the normal leucocyte.

3. Leucocyte AA diffuses slowly to the surrounding medium.
4. A maximum concentration of leucocyte AA was demonstrated in the same leucocyte population exposed to different concentrations of DHA.
5. The entry of DHA into and its reduction by human leucocytes are independent processes.
6. Sulphydryl compounds, possibly GSH, are involved in the leucocytic reduction of DHA.
7. Neither leucocyte membranes nor lysed leucocytes could be shown to reduce DHA even in the presence of added GSH under the experimental conditions used.
8. Leukaemic leucocytes do not contain raised amounts of GSH.

Part II

The Determination of Leucocyte AA in
Post-surgical Patients and in other clinical conditions.

Introduction

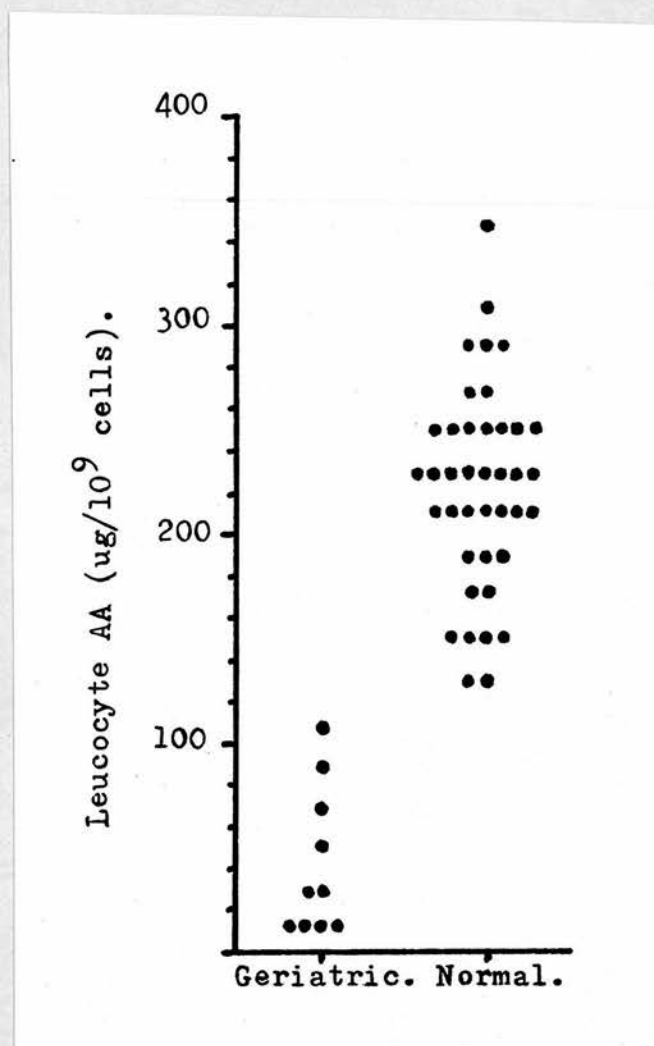
As a preliminary to the study of the effect of surgery on leucocyte AA the normal range for the vitamin C content of these cells had to be established. By reference to this range, it can be determined whether the patient's leucocyte level of AA was within or below normal prior to surgery, and also whether surgery could depress tissue AA below the lower limit of normal. TAA determinations always gave similar results to those of AA in the metabolic experiments; because of this, only TAA assays were carried out in the following studies.

The subjects chosen to donate blood for the construction of the normal range were all between the ages of 18 and 35, and were either laboratory staff or medical staff. Leucocyte TAA was determined in forty normal individuals (34 male and 6 female) and the results subjected to statistical analysis. No attempt was made to determine separate normal ranges for males and females due to the small number of values available for women in the present study. The mean value of these forty determinations was $239 \mu\text{g.}/10^9$ leucocytes, with a standard deviation of 52, giving a normal range of 135 - 343 (Mean \pm 2SD) $\mu\text{g.}/10^9$ leucocytes.

Geriatric Investigations

In the course of clinical investigations carried out since 1960, as part of the work of this thesis, it has been

FIGURE 11.



**Distribution of leucocyte AA concentration
in normal and in geriatric subjects.**

found that most elderly subjects possess leucocyte AA values which are below the lower limit of the normal range established previously. White blood cell AA was estimated in ten consecutive admissions for geriatric assessment. The distribution of the vitamin concentration in the normal and geriatric groups are compared in Figure 11.

White blood cell vitamin C levels were also investigated in the two pathological conditions of leukaemia and scurvy, prior to studies on surgical cases.

Scurvy:

Investigations of suspected cases of hypovitaminosis C demonstrate the value of the estimation of leucocyte AA levels for immediate assessment of the nutritional status of a subject. The procedure was as follows:-

20 ml. of fasting blood was withdrawn for white cell AA estimation. Following this, saturation tests were carried out on these subjects by the method of Harris and Abbasy (1937), which is used in many hospitals as an aid in the diagnosis of scurvy. Results are shown in Table 7, where the initial leucocyte level is related to the number of consecutive days during which a standard dosage of vitamin C (11 mg./Kg. body weight) had to be administered before saturation of the seven subjects was achieved, in contrast to the two-day maximum period required for saturation to occur in normal subjects.

TABLE 7.

Investigation of leucocyte AA in patients
suspected of having some degree of hypovitaminosis C

<u>Case</u>	<u>Age</u>	<u>Leucocyte AA</u>	<u>No. of days to effect stn. using</u> <u>Std. test dose of 11mg./kg.daily</u>
1 ♂	74	51	6
2 ♂	67	20	8
3 ♀	81	91	* >5
4 ♀	65	156	4
5 ♀	75	177	3
6 ♀	90	48	7
7 ♂	91	20	10
8 ♀	80	158	2
9 ♂	81	40	6
10 ♂	65	65	5

* No final saturation time could be performed on this patient who died the following day. Post mortem findings were haemorrhagic cystitis with evidence of nephritis.

Leukaemia:

The investigation of five cases of myeloid leukaemia showed that all had low leucocyte AA levels (Table 8). A saturation test carried out on one of these cases took five days by the standard procedure, indicating that some degree of deficiency existed.

Surgical Investigations.

Procedure:-

White blood cell AA was determined in six patients undergoing elective surgery for a variety of conditions, (a) just prior to operation; (b) 48 hours after operation, and (c) in three cases one week after operation (Figure 12).

A significant post-surgical fall in leucocyte AA after 48 hours was demonstrated in four of the six cases investigated. The three subjects whose recovery was followed in the ensuing five day period showed a slow increase in leucocyte AA concentration.

Discussion.

General:

In Appendix I are assembled the normal ranges of leucocyte^{AA} concentrations according to different authors. The early investigators who expressed their results in terms of wet weight or mg./100 ml. of cells, included an unknown proportion of non-leucocyte material, and so underestimated the vitamin content. This underestimation was, by chance,

TABLE 8.

Investigation of leucocyte AA in cases
of myeloid leukaemia

<u>Case</u>	<u>Age</u>	<u>myeloid leukaemic type</u>	<u>ug.AA/10⁹ cells</u>
A.C.	34	acute	22
*M.M.	45	chronic	74
" A.P.	33	chronic	73
♂ A.McC.	61	chronic	68
*A.G.	64	acute	75

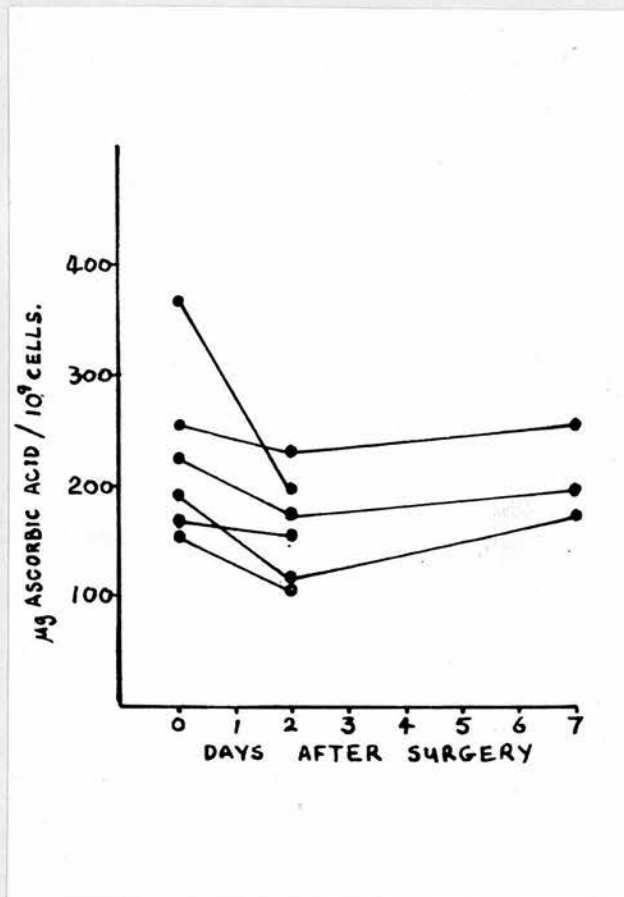
* Patient from M.R.C. unit,
Western General Hospital.
Dr. Baikie.

♂ Patient from City Hospital,
Edinburgh.

" Saturation test performed
requiring 5 days by the
method of Harris and Abbasy(1937).

FIGURE 12.

The response of leucocyte AA to
surgical trauma



offset by the use of dichlorophenol-indophenol under conditions which were non-specific for AA, other reducing substances such as GSH being estimated also, so that overestimation in the analytical procedure yielded final results which were very similar to those obtained currently by more exact techniques.

The much wider range obtained by Denson and Bowers (1961) is due to the fact that these authors made no allowance for the presence of platelets, but related the amount of TAA estimated to the number of white cells present. This practice, as can be seen by reference to the experimental section, can result in overestimating the leucocyte AA concentration by 100% if normal numbers of leucocytes and platelets are present.

The upper limit of the normal range given by Barkhan and Howard (1958) is lower than that found in the present work. This discrepancy may be due to the fact that the process of isolation of pure leucocyte suspensions employed by these investigators occupied a total time of 4 hours. Even after 1 hour, as shown in Table 1, AA begins to diffuse from the leucocyte. After a lapse of 4 hours before analysis therefore, it would be expected that the AA content of the cells would be lower than the true value.

Saturation tests cannot have rapid effects in increasing the concentration of leucocyte AA since the vitamin

appears in the blood stream after oral ingestion in the reduced form, in which condition it cannot penetrate the cell membrane. The limiting factor in the increase of leucocyte AA is therefore the rate of oxidation of AA to DHA in vivo, which is probably not constant, since vitamin C has been shown to leave the adrenal gland as DHA after stimulation of the adrenals by ACTH (Salomon, 1957).

Effect of Age on Leucocyte Concentrations.

The decrease of leucocyte AA with age which is shown in Figure 11 is striking. None of the patients, however, showed any clinical signs of hypovitaminosis C and the four subjects having the lowest levels could not be distinguished by means of leucocyte AA concentrations from those patients in Table 7 having equally low values, and yet who exhibited scorbutic symptoms.

Several authors have noted a difference in leucocyte levels between older and younger age groups. Borlina (1957) studied vitamin C in the senile and found that, on giving the vitamin orally, maximum serum values were attained only after periods greater than 14 days in contrast to the 5 - 7 days of younger age groups. Borlina also found that, if senile subjects were deprived of the vitamin after the maximum serum values were attained, the serum concentration fell to low values after only 5 - 7 days, whereas the younger group showed persistence of high values for 2 - 3 weeks. These

observations could be explained either by an increased catabolism of the vitamin by the elderly or a decreased capacity to retain it. In either case, increased amounts of the vitamin were necessary in the senile patient to maintain the same blood levels as those of younger subjects. Denes (1962) confirmed the work of Borlina and noted deficient serum levels in prematurely senile subjects, in spite of their taking adequate oral supplements. A statistically significant decrease of serum vitamin C with age was found by Kirk (1953) and Vaishwanar (1959) in extensive studies covering a population of over 300 subjects. The low white cell content of the vitamin observed in the aged would seem to be a reflection of the greatly diminished levels of AA present in their blood plasma, for the leucocyte is probably dependent for its AA upon the small concentration of DHA produced by oxidation of plasma AA.

There is also evidence to suggest that the low leucocyte levels of the aged are a result of decreasing endocrine function. It has been shown that corticosteroids activate an AA concentrating mechanism in vitro (Van and Hill, 1958) and that cortisone and hydrocortisone incubated with rat liver slices in glucose/bicarbonate buffer strikingly decrease the diffusion of AA from the tissue.

Denson and Bowers (1961) also observed low white cell AA levels in the elderly and gave a range of

20 - 36 $\mu\text{g.}/10^9$ cells from a series of 50 geriatric patients. They found that the low AA levels observed in geriatric subjects were not related to the excretion of p-hydroxyphenylacetic acid (PHPA), which could not be detected in the urine of the patients investigated. This was unexpected, for Dobriner, Lavin and Rhoads (1942) had found abnormally large amounts of phenolic acids in the urine of vitamin C deficient patients. The work of Dobriner et al was extended by Boscott (1954), who showed that PHPA present in the urine of patients with steatorrhoea disappeared when an abnormally high intake of vitamin C was given. Zannoni (1960) later showed that vitamin C produced its effect in these steatorrhoea patients by protecting PHPA oxidase from inhibition by its substrate, thereby allowing the catabolism of PHPA, an intermediate metabolite of tyrosine, to proceed normally. A possible explanation of the failure of Denson and Bowers to find any PHPA excretion in the elderly is that, in spite of low tissue levels of AA, sufficient protection is afforded PHPA oxidase for the enzyme to deal adequately with the tyrosine when metabolism is diminished in the aged, and it would be interesting to discover whether surgery on elderly patients possessing leucocyte AA levels of 0 - 20 $\mu\text{g.}/10^9$ cells could produce a phenolic aciduria.

The low levels of leucocyte AA observed in geriatric

patients may be due primarily to poor dietary habits of economic origin, or to senile indifference, and any range of normal values must take into account the age of the population examined. Since no deficiency signs were observed in the geriatric cases of Figure 11 we are forced to the conclusion that the finding of a leucocyte AA value of $20 - 140 \mu\text{g.}/10^9$ cells in an elderly patient does not prove that a deficiency state exists. If the leucocyte AA concentration is in the lower range of these values however, a deficiency potentially exists, for Crandon (1961) has shown that there is a statistically significant increase in the occurrence of post-surgical wound dehiscence in patients whose pre-operative leucocyte AA concentrations are below $8 \text{ mg.}/100 \text{ ml.}$ ($70 \mu\text{g.}/10^9$ cells).

Clinical Investigation of Scurvy.

Detectable leucocyte AA levels are of clinical value in the exclusion of scurvy.

The finding of very low levels of leucocyte AA, when taken in conjunction with clinical evidence, can substantiate a diagnosis of scurvy, but it cannot alone justify such a diagnosis. This was shown by the Medical Research Council's study during World War II. Individuals receiving only 10 mg. of vitamin C per day over a period, although protected from all clinical signs of scurvy, could not be satisfactorily differentiated chemically from scorbutic subjects,

for the leucocyte concentrations of both groups were below the minimum reliable figure of 2 mg.% ($20 \mu\text{g.}/10^9$ cells).

Clinical Investigations of Myeloid Leukaemia

The findings of low leucocyte AA levels in the five cases of myeloid leukaemia in Table 8 is in agreement with the results of Waldo and Zipf (1955); and Bodansky, Wroblewski and Markardt (1952), who found low values in acute and chronic lymphatic and myeloid leukaemias. Barkhan and Howard (1958), however, found that the AA content of leucocytes in the leukaemias was not always low, and confirmed the earlier report of Cuttle (1938) that the leukaemic subject takes longer to saturate than normal, which has been confirmed in the present study. It seems likely that the low plasma levels of AA observed in leukaemia (Hagtvet, 1945; Waldo and Zipf, 1955) are responsible for the accompanying low leucocyte AA values, as was suggested previously to account for the similar findings in elderly subjects. However, there are alternative explanations for the low leucocyte AA values of leukaemic subjects which will now be discussed.

Although no specific enzymatic reducing system for DHA is known in animal tissues, it seems likely that an enzyme is involved, for Thomson *et al* (1956) have shown that the DHA reducing system of human erythrocytes is heat labile.

The DHA-reductase system can be regarded as an AA preserving mechanism. If DHA is not reduced, it undergoes irreversible oxidation to products having no vitamin C activity. The low plasma levels of AA reported in pernicious anaemia (Freeman and Hofkesbring, 1957; Todd, 1959) led Hughes and Kilpatrick (1964) to suggest that this was due to a decreased erythrocyte DHA-reductase activity, but in fact they found an increased activity of this system; this was surprising in view of Jocelyn's (1960) claim that vitamin B₁₂ controls the activity of the enzyme GSH-reductase. Since other authors had reported increased activities of erythrocyte arginase (Reynolds, Follette and Valentine, 1957), alkaline phosphatase (Valentine, Kovichi and Fredericks, 1961) and the enzyme synthesising aminolaevulinic acid (Laver, Neuberger and Udenfreind, 1958) in pernicious anaemia, Hughes and Kilpatrick (1964) concluded that the increase in DHA-reductase activity is not a specific effect of this disease but is part of a generalised response by the erythrocyte enzyme system to a reduction in the red cell count. If this is so, it is possible that the reverse circumstances could obtain, in which an increase in the number of cells, as in the leukaemias, could result in a decrease of their DHA-reductase activity; this would account for the low leucocyte AA levels recorded in Table 8.

The imperfect nature of the leukaemic cell and its

consequent shorter functional life (Whitby and Britton, 1963) might also be a factor which would be expected to reduce this type of cell's enzymic activity.

Whatever the cause of the low leucocyte AA levels in the leukaemias proves to be, the rapid reduction of added DHA seen in the metabolic experiments described earlier makes it unlikely to be a decreased DHA-reductase activity, since there are sufficient reserves of the enzyme to deal with relatively large amounts of DHA when present.

The finding of low leucocyte GSH values in myeloid leukaemia in the present work (Table 6), existing concurrently with low AA values, possibly indicates a functional relationship between these two compounds; this was suggested in Part I.

Surgical Investigations

In Figure 12 it can be seen that in all six surgical subjects a fall was demonstrable in leucocyte AA after 48 hours but only in four cases was the fall significant. Studies were continued on three of these subjects for a further period of five days after which time an increase in leucocyte AA could be detected although pre-operative levels had not been reached.

While the work described here was in progress, Grandon, Lennihan and Reif (1961) published their findings on the effect of surgery on plasma and leucocyte AA in 150

patients. They carried out determinations pre-operatively, at the end of the surgical procedure, and in some cases 24 hours after surgery, expressing results for the white cells as mg./100 ml. buffy coat. Crandon et al (1961) demonstrated an average fall of 20% in buffy coat AA levels after surgery, often within as short a period as 1 hour. After 24 hours the white cell level remained at the immediate post-surgical value or decreased slightly. In these studies no correlation between the degree of fall, the type and duration of the operation, or the type of anaesthesia could be found. There was an eightfold higher rate of wound dehiscence in those patients with deficient blood AA levels (less than 0.2 mg./100 ml. in plasma, or less than 8 mg./100 ml. buffy coat [$70 \mu\text{g.}/10^9$ cells]) at the primary operation, compared to those with adequate levels.

In a previous paper, Crandon, Mikal and Landau (1952) described vitamin C metabolism in the post-surgical patient as being in a "state of flux". They were referring to the fact that, in the post-surgical patients studied, high plasma AA values were sometimes found in association with low values for the buffy layer, whereas in the normal subject either both were high or the plasma concentration was low and the buffy layer was high. Crandon et al (1952) state that in surgical cases where the vitamin concentration in plasma was high while that of the buffy layer was low, oral supplements of vitamin C had been prescribed.

In the light of the results of **Part I** of this thesis Crandon's (1952) finding of elevated plasma in the presence of low buffy coat AA values was presumably due to the recently administered vitamin; this would not be absorbed by the white cells, and would therefore not immediately increase the AA of the white cell layer. In these subjects, ingestion of vitamin C would increase the plasma concentration of AA to a high value while the leucocyte AA would remain low, but given time, the plasma levels would stabilise at a value just below the renal threshold value of 1.3 mg./100 ml. It is suggested, therefore, that the "state of flux" referred to by Crandon et al (1952) does not exist.

The results shown in Figure 12 indicate a decrease of leucocyte AA similar to that demonstrated by Crandon, with a subsequent increase occurring somewhere between 2 and 7 days after surgery.

Crandon et al (1961) noted that the post-surgical fall in leucocyte AA was most clearly seen in patients having high pre-operative concentrations (see Figure 12). This occurrence is to be expected since the higher the concentration of a compound, the more obvious will be its decrease in concentration after a sudden increase in metabolic use. On the other hand, if the concentration of a compound is very low prior to an increase in its requirement, the decrease in concentration may not be detectable. In fact, part of the research

programme of the present thesis had to be abandoned because the leucocyte AA values were often on the borderline of detection ($20 \mu\text{g.}/10^9$ cells) in a group of elderly patients who were to undergo pinning of the femur after accidental fracture, so that no response to surgery could be recorded. It is notable, however, that trauma was already present in these cases, a condition which will be shown later in this thesis to lower the vitamin C nutritional status of the subject.

Summary of Results in Part II

1. A normal range for leucocyte AA has been determined.
2. The levels of AA in the leucocytes of elderly patients are predominantly in the lower part of this range.
3. It has been confirmed that in acute and chronic myeloid leukaemia the white cell level of AA is low.
4. A post-surgical decrease in leucocyte AA has been demonstrated, and the post-surgical findings of others discussed in relation to the results of the metabolic experiments in the first part of this thesis.

RESULTS

Part III

The use of Vitamin C Tolerance Tests in the Detection
of Increased Requirements of the vitamin after Surgery

General Introduction

Although measurements of leucocyte AA allow the status of tissue AA to be assessed from time to time without exerting a modifying effect on subsequent analyses, the possible value of consecutive tests of a tolerance type was considered worthy of investigation. Evidence for increased vitamin C requirements after surgery was sought in those subjects in whom, because of very low leucocyte AA levels, such information could not be obtained from the studies described in the previous section. It is possible that the response to surgery reported in Part II of this thesis, and also by Crandon et al (1961), might be restricted to the leucocytes only, but the response to a vitamin C tolerance test would reflect a summation of response in all tissues. It was also considered that the very large leucocytosis which Pepper and Lindsay (1960) found to be induced by surgery, cast some doubt on the validity of the leucocyte AA concentration shortly after surgical procedure.

Other objections to the use of leucocyte AA levels in these studies had also become apparent. The technical manipulations necessary for one estimation require six hours work, and the completion of more than two tests in one day is beyond the capacity of a single worker. The volume of blood required (20 ml.) for the estimation may be difficult or even impossible to obtain from post-surgical patients.

if they are seriously shocked. For these reasons, it was felt that a technically simpler test was desirable.

Introduction.

The oral AA tolerance test was first suggested by Lund, Lieck and Clemenson (1937). They observed that, several hours after a test dose of AA, the plasma level in well nourished individuals was much higher than in deficient subjects. This work was extended by Butler and Cushman (1940), and by Kajdi, Light and Kajdi (1939). Stotz, Shinnars and Chittick (1942), and Rinehart and Greenberg (1942) were able to classify responses to a loading dose of the vitamin into four groups, which will be discussed later. Dutra, Pearson and Darby (1959) investigated the usefulness of AA tolerance tests in the diagnosis of scurvy.

The purpose in applying these AA tolerance tests is to gain some insight into the adequacy of the vitamin C content of the tissues. Since all plasma vitamin C seems to be in the free state, a reliable index of its sufficiency in the body should be furnished by its rate of penetration from the plasma into the tissues. After oral ingestion, therefore, if the vitamin C content of the tissues is low, the rapid flow of vitamin from the plasma to the tissues would result in only a small rise of the plasma concentration. Conversely, the more adequate the tissue vitamin concentration, the bigger the increase in the plasma. This

being the case, the second of two tolerance curves carried out on the same subject within 48 hours, should normally produce higher plasma values than the first, since the first test will have made the subject more nearly saturated with vitamin C than before. This has been confirmed by experiment. (Figure 13). When such consecutive tolerance tests were carried out on surgical patients, it was planned to perform the first test pre-operatively and the second 48 hours after surgery. In spite of the partially-saturating effect of the first test dose on normal subjects (Figure 13), it was expected that the post-surgical increase in the metabolic requirement of vitamin C would be great enough to be reflected by decreased levels of plasma AA in the second tolerance test performed 48 hours after surgery. This would provide evidence of a more rapid flow of the vitamin from the plasma to the tissues due to the increased requirement of the latter.

Intravenous administration of the vitamin has been suggested by some authors (Wright, Lillienfeld and MacLenathan, 1937; Finkle, 1937). This would eliminate possible errors due to differences in intestinal absorption rates, but Van Eekelen and Heinemann (1938), and Stotz et al (1942) pointed out that the intravenous route causes the renal threshold to be greatly exceeded and results in loss of a large proportion of the vitamin dose; this must influence the blood levels

observed subsequently.

The oral tolerance test was adopted in the present investigation. The loading dose used has been varied by different workers. Rhinehart et al (1942); Wolfer, Farmer and Manshardt (1947); Dutra et al (1959), all used a dose of 15 mg./Kg. body weight. The modified test of the last-named authors was designed particularly for the diagnosis of scurvy, and so employed the large loading dose, which has been used in the present studies on suspected scorbutics. In the investigation of surgical patients, however, a smaller dosage was given (6 mg./Kg. body weight) as used by Stotz, Shimmers and Chittick (1942). This smaller intake rarely results in more than 10 per cent. being excreted in the urine in the five hour test, and hence the principal factor in determining the difference between curves will be the rate of absorption of the vitamin from the plasma by the tissues. Another reason for using the smaller dose was the fact that the first test would be less likely to produce a degree of vitamin C saturation that would seriously obscure the effect of surgery as measured in the second test.

The main factor causing error in such tolerance tests is the variable rate of intestinal absorption. Information gained about tissue stores from successive tolerance curves in the same patient will be worthless if the absorption rate varies greatly.

When discussing the interpretation of results of their modified vitamin C tolerance test, Dutra et al (1959) stressed this reservation as to the usefulness of such tests; they stated that a maximum rise of plasma AA to concentrations below 0.25 mg./100 ml. is consistent with a diagnosis of scurvy, but such extremely low maximum values may be encountered either in states of severe depletion of tissue stores, or in marked degrees of malabsorption.

It is therefore necessary to take account of the rate of intestinal absorption when consecutive curves are compared. If the maxima in plasma concentration occur about the same time after oral ingestion, the two curves are comparable and can furnish accurate information on relative levels of tissue reserves in the patient at the time of the test. The interpretation of consecutive tolerance curves is uncertain if the plasma maxima occur at markedly different times; under these circumstances variation in the rate of absorption from the alimentary tract is to some extent responsible for the difference in the shape of the curves.

After both the 15 mg./Kg. dose of Rhinehart and Greenberg (1942) and the 6 mg./Kg. supplement of Stotz et al (1942), the maximum plasma concentration normally occurs in the 3 hr. specimen. This has been confirmed in the course of studies (Table 9) but, in initial investigations involving post-surgical patients, it was sometimes found that the

plasma concentration was still increasing after 4 hrs. The delay in reaching maximum values could be explained either by a slower rate of intestinal absorption alone, or by a combination of this and an increase in vitamin requirement. These two processes would have an effect on the curve indistinguishable from each other and in these circumstances it would be impossible to say if a post-surgical increase in vitamin requirement has taken place or not.

In the following experiments an attempt has been made to correct automatically for variable intestinal absorption rates during serial vitamin C tolerance tests by giving a standard dose of D-xylose simultaneously with the vitamin loads. The oral dose of D-xylose most often used in the clinical diagnosis of malabsorption syndrome is 25 g. (Fourman, 1948; Gardner and Perez-Santiago, 1956). The capacity of the intestine to absorb D-xylose was studied by Morgan and Sammons (1964) who administered eight different dosage levels, varying from 0.5 - 25 g., to five normal subjects. When 5 g. or less was given, the percentage of the dose which was excreted by the kidneys in five hours was constant. When the dosage was above 5 g., the percentage excreted was smaller. This suggested that amounts greater than 5 g. exceeded the absorptive capacity of the intestine, and would explain the diarrhoea commonly observed after a 25 g. dose. The possibility of nausea and diarrhoea in

post-surgical patients was naturally undesirable and so a 5 g. dose was chosen. 5 g. D-xylose has been employed by Butterworth, Perez-Santiago, Martinez de Jesus and Santini (1959); Santini, Sheehy and Martinez de Jesus (1961); Hubble and Littlejohn (1963), and by Joske and Haagensohn (1964). No nausea or diarrhoea has been observed in tests using 5 g. D-xylose.

Both vitamin C and D-xylose are absorbed high in the intestinal tract (Todhunter, Robbins and McIntosh, 1942; Campbell and Morrison, 1963). After absorption, the tissues would be expected to take up AA at a rate depending on their requirement, so that the blood AA level depends both on tissue requirement, and on its rate of absorption from the intestine. The blood xylose concentration also depends on the rate of absorption, and in addition on renal clearance, which is a constant since there is no tubular reabsorption of this compound. After simultaneous administration therefore, if the blood concentrations of both are determined after a given interval and the result expressed as a D-xylose/ascorbate ratio, this will give an expression, independent of the rate of intestinal absorption, which will represent the avidity of the somatic cells for vitamin C. In any subsequent tolerance test on the same patient an increased value in this ratio, after the same time interval, would indicate an increased metabolic requirement for the vitamin.

Results

Normal Response to AA tolerance test

An initial series of investigations into the response of normal individuals to orally administered vitamin C confirmed the occurrence of three hour maximum blood levels (Table 9). Only in the initial investigations were AA as well as TAA determinations carried out. These studies showed the presence of small amounts of DHA in normal human blood. The observation of other workers was confirmed that the original fasting difference existing between the oxidised and reduced forms remains constant during the test. The proportion of DHA to AA found, however, was never greater than 10%, and in many subjects the TAA and AA determinations gave identical results. Only TAA was determined in subsequent studies.

Scorbutic Response to Vitamin C

Table 10 shows the results of investigations on eight patients for suspected scurvy. Clinical evidence for this condition was present in all cases. The leucocyte AA value of the patient was determined, followed by a vitamin C tolerance test (dosage 15 mg./Kg.) in which specimens of blood were taken for analysis before the ingestion of the vitamin dose, and again after three and four hours. Saturation of the patient was then carried out by the method of Harris and Abbasy (1937) and the number of days taken to

TABLE 9

Ascorbic Acid Tolerance Curves of Normal Subjects

Subject	Age	Oral dose of AA given (mg./Kg. body wt.)	TAA Plasma Concentration(mg./100ml)				
			Fasting	1 hr.	2 hr.	3 hr.	4 hr.
P.H. 0	36	15	0.3	0.56	1.48	2.13	1.9
J.S. 0	22	15	0.4	1.1	1.68	1.72	1.55
S.H. 0	26	15	0.56	1.5	2.3	2.6	2.3
J.H. 0	38	6	0.2	0.55	0.83	1.13	1.0
H.C. 0	45	6	0.3	0.63	0.88	1.58	1.18
F.M. 0	27	6	0.1	0.25	0.55	1.1	0.72

The test was begun at 8 a.m. with all subjects in the fasting state, when approximately 3 ml. blood was taken and added to a sequestrene container in ice. All specimens were transported to the laboratory without delay where centrifugation, followed by deproteinisation of the plasma in the appropriate acid, were carried out within 10 minutes. Oral doses of AA were then given in 250 ml. water and hourly specimens of blood removed for the following 4 hours.

TABLE 10

Patient	Plasma TAA Concentrations during the test (mg./100 ml)				Leucocyte AA concentration ug/10 ⁹ cells	No. of days on Vitamin C supple- ment required to produce satura- tion
		F	3 hr.	4 hr.		
E.K. ♀	(a)	0.1	0.23	0.12	66	12
Age 83	(b)	1.1	1.93	1.9	158	-
W.M. ♂	(a)	0.1	0.8	0.68	84	5
Age 87	(b)	0.4	1.7	1.7	174	-
D.O. ♂	(a)	0.1	0.18	0.18	40	9
Age 79	(b)	0.8	2.16	2.25	160	-
G.C. ♂	(a)	0.1	0.1	0.1	20	8
Age 85	(b)	0.7	1.84	2.06	204	-
J.M. ♂	(a)	0.1	0.20	0.17	58	9
Age 79	(b)	0.9	1.8	1.6	178	-
R.U. ♂	(a)	0.1	0.32	0.37	109	5
Age 68	(b)	0.5	1.6	1.54	200	-
P.C. ♂	(a)	0.28	1.58	1.18	296	1
Age 45	(b)	-	-	-		
J.H. ♂	(a)	0.1	0.2	0.2	20	10
Age 87	(b)	1.4	1.4	2.1	-	-

(a) Pre-saturation values

(b) Post-saturation values

* The response to vitamin C of suspected scorbutic patients before and after saturation with the vitamin, and the relation of these results to leucocyte AA levels and the number of days required to produce saturation according to Harris and Abbasy (1937).

achieve this noted. The day following saturation a second tolerance test was carried out, and the white blood cell AA level determined.

Vitamin C Status of Geriatric Patients as determined by a Vitamin C Tolerance Test

Table 11 shows the results of vitamin C tolerance tests (15 mg./Kg.) carried out on ten patients who had been admitted to the medical wards for routine geriatric assessment.

Normal Response to two Consecutive Doses of Vitamin C

Figure 13 shows the response of a normal subject to successive doses of vitamin C as measured by the concentration of TAA in the plasma. The tests were performed with an interval of 48 hours as was the case in the later surgical investigations. The second test attained higher concentrations due to the effect of the first test-dose in partially saturating the subject's tissues.

The Effect of Surgery on Vitamin C Tolerance

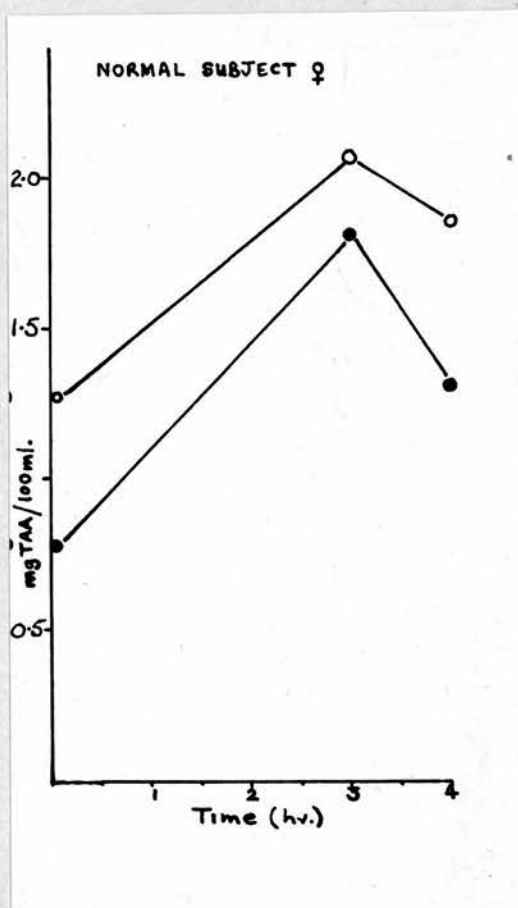
In a series of 12 patients, pre-surgical tolerance curves were compared with the response from the same patient 48 hours after operation. These results are shown in Figures 14 - 25. Brief case histories are included with each figure as well as details of the individual experiment and post-surgical course.

TABLE 11.

Vitamin C Tolerance Tests
on Random Geriatric Patients.

<u>Patient</u>	<u>Plasma levels of TAA (mg./100 ml.)</u>		
	<u>Fasting</u>	<u>3 hr.</u>	<u>4 hr.</u>
I.M.	<0.1	0.20	0.18
S.M.	<0.1	0.31	0.38
P.Bu.	0.26	0.91	0.90
L.L.	<0.1	0.24	0.16
I.B.	0.10	0.40	0.46
P.L.	0.32	1.27	1.08
I.C.	<0.1	0.40	0.30
J.M.	0.12	0.24	0.25
P.Be.	<0.1	0.43	0.28
W.H.	<0.1	0.18	0.13

FIGURE 13.



Normal response to two doses of AA (6mg./kilogram).

The interval between the tests is 48 hours.

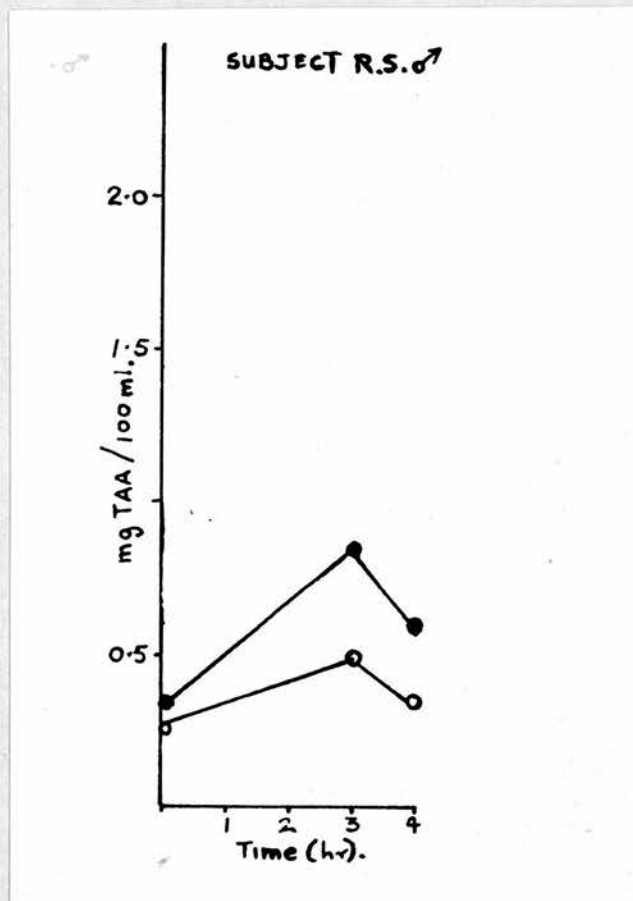
- 1st AA tolerance test.
- 2nd AA tolerance test.

Figures 14 - 25.

Vitamin C Tolerance Tests on Surgical Patients

- The initial tolerance curve carried out prior to surgery.
- The second tolerance test, which was performed in every case following surgery after an interval of 48 hours.
- ▲ The third tolerance test was carried out only in certain cases and at times which were dictated by circumstances. These are explained in individual legends.

FIGURE 14.



CASE 1.

Subject R.S. ♂ Age 76. Operative procedure:- Prostatectomy.

History:- 28/3/64. Admitted with chest pain and breathlessness. Had very good health until 1962 when he developed hypertensive retinopathy.

Relevant notes:- Lives with wife who is now senile. Both looked after by daughter-in-law and district nurse. Appetite good.

Diagnosis - Pulmonary embolism. Also had enlarged prostate.

7/4/64 7 a.m. Abdominal pain, developed urinary retention.

12/4 Transferred to Surgical ward.

16/4/64 1st Vit. C tolerance test.

17/4 Operation : Prostatectomy. Anaesthetic: Pentothal, Nitrous
oxide, oxygen.

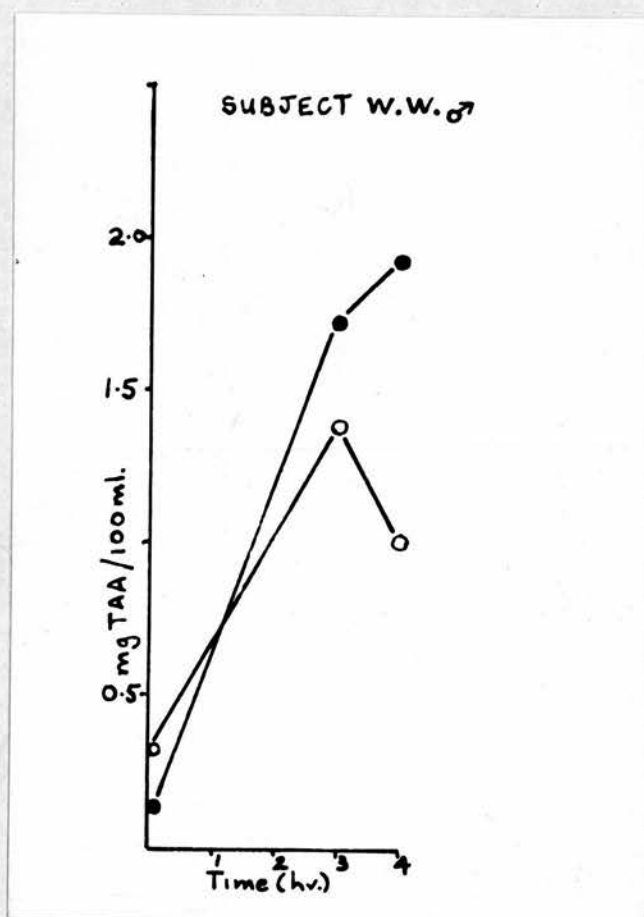
19/4 2nd Vit. C tolerance test.

22/4 Wound dressed, 1 stitch cut out. No discharge or redness or
induration. Wound appeared weakened. Probable separation
of recti.

27/4 Small pressure sores on sacrum.

3/5 Developed left hemiplegia and remained comatose until he died
on 9/5. Post mortem showed cerebral haemorrhage, infarction
of the cerebral capsule and softening of the adjacent brain
substance.

FIGURE 15.



CASE 2.

Subject W.W. ♂ Age 74. Operative procedure:- Prostatectomy.

History: Admitted 5/11/63. Difficulty in starting micturition for 3 months.

Large prostate present. General condition good.

Relevant notes: Appetite good. Lives with his wife. Both well.

12/11/63 1st Vit. C tolerance test.

13/11 Operation. Anaesthetic: Pentothal, Nitrous oxide and halothane.
Supra-pubic incision. Enlargement of both lateral lobes of prostate which was easily removed. Wound closed in layers.

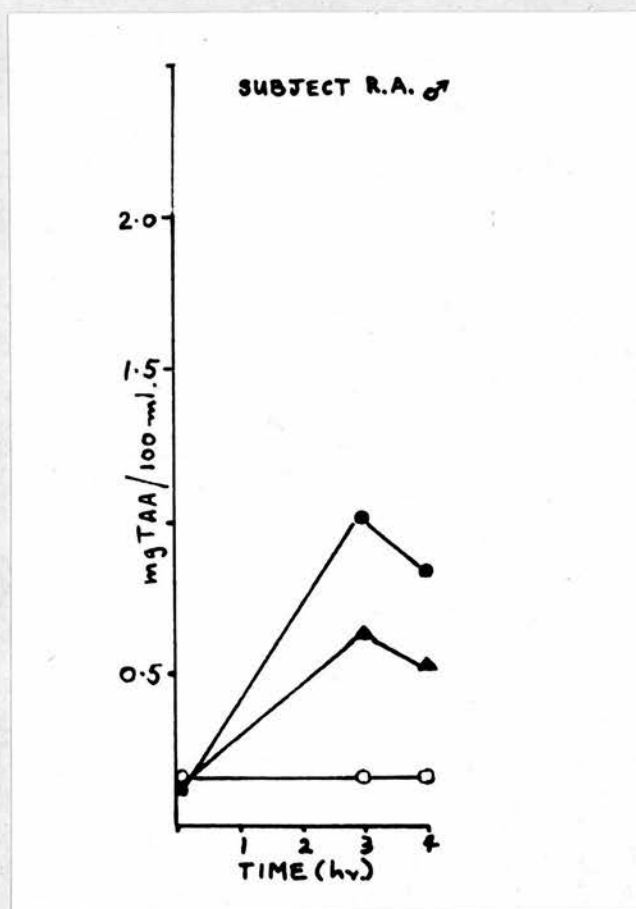
14/11 Drain normal.

15/11 2nd Vit. C test completed.

20/11 Up and about.

22/11 Stitches out. Wound well healed. Discharged.

FIGURE 16.



CASE 3.

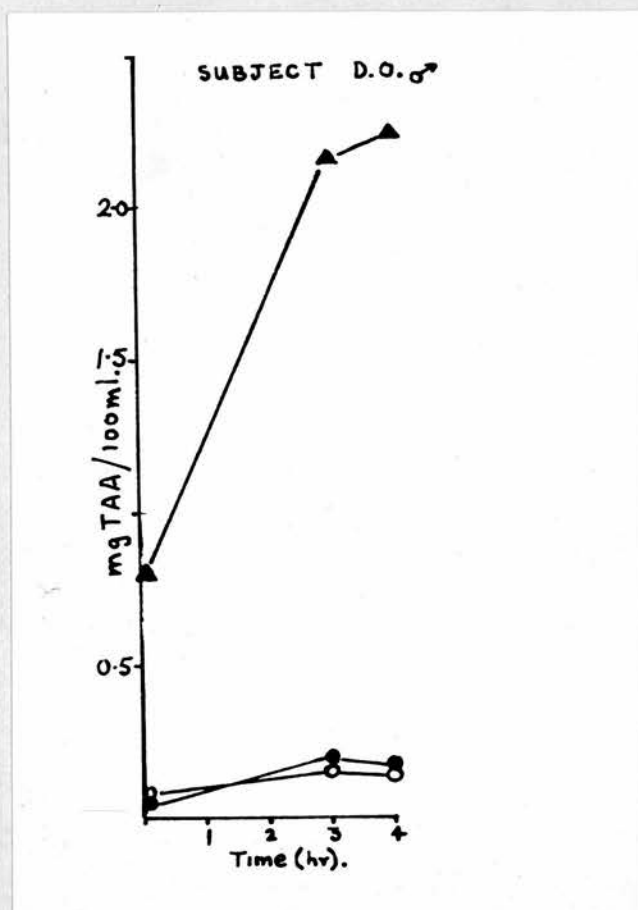
Subject R.A. ♂ Age 55. Operative procedure:- Partial gastrectomy.

History: Experienced acute pain in epigastrium.

Relevant notes:- Appetite fairly good. Weight loss $1\frac{1}{2}$ stones in previous 2/52.

- 21/11/63 Admitted. Hb 82%.
- 22/11 1st Vit. C tolerance test.
- 23/11 Operation. Partial gastrectomy performed for cancer of stomach.
Anaesthetic:- Pentothal.
- 25/11 8 a.m. 2nd Vit. C tolerance test. Later abdomen became distended below umbilicus.
8 p.m. No bowel sounds - drip and suction because of paralytic ileus (see Fig. 16, 2nd test).
- 26/11 Abdomen softer.
- 27/11 3rd Vit. C tolerance test.
- 4/12 Up and about - still some discharge from middle of wound.
- 5/12 Stitches out. Hb 75%.
- 10/12 Discharged.

FIGURE 17.



CASE 4.

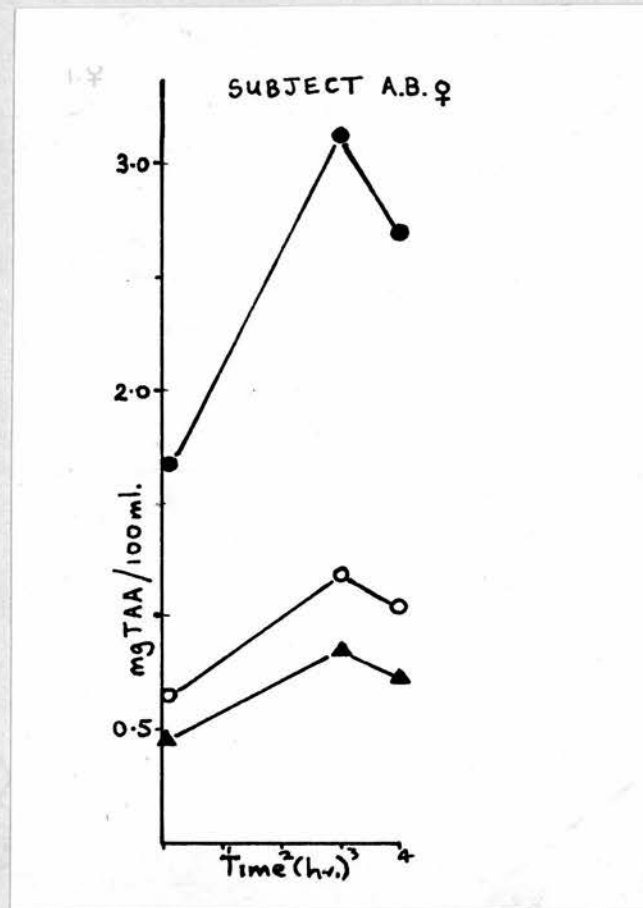
Subject D.O. ♂ Age 79. Operative procedure:- Cruciate incision of abscess.

History:- 1/60. Above knee amputation (R) for peripheral vascular disease. 3/63. Amputation (L) leg for similar condition. After discharge home, developed bedsore on left buttock and slight discharge from part of stump wound.

Relevant notes:- Lives with married daughter. Appetite poor. Dislikes vegetables and fruit.

- 17/5 Admitted for geriatric assessment and treatment of bedsore. Abscessed bedsore 4 x 4 cm.
- 28/7 1st Vit. C tolerance test.
- 29/7 Cruciate incision of L. sacral abscess (2 cm. deep).
- 31/7 2nd Vit. C tolerance test and leucocyte level of ascorbic acid showed deficiency. Saturation test started.
- 9/8 Patient saturated with respect to Vit. C. Kept on 100 mg. t.i.d.
- 10/8 3rd Vit. C tolerance test.
- 29/8 Sacral sore 3 x 2.5 cm. (also more shallow).
- 12/9 " " 2 x 1.5 cm.
- 27/9 " " 1 x 1 cm.
- 14/10 " " healed.

FIGURE 18



CASE 5.

Subject A.B. ♀ Age 55. Operative procedure:- Reduction of hiatus hernia.

History:- Barium meal a year previously showed a transient para-oesophageal hernia of the fundus of the stomach, spontaneously reducing itself in the erect position. Later developed dysphagia to solids. Barium meal in 8/63 showed large hiatus hernia not reducing itself.

Relevant notes:- The patient ate light meals, and enjoyed vegetables and fruit.

9/1/64 Admitted. Hb 71%. 1st Vit. C tolerance test.

10/1 Operation. Hernia easily reduced by 3 linear sutures. Splenectomy had to be carried out because of bleeding. Anaesthetic:- Pentothal.

12/1 2nd Vit. C tolerance test.

14/1 Hb 75%, started on iron and vitamins (including vitamin C 50 mg. t.i.d.)

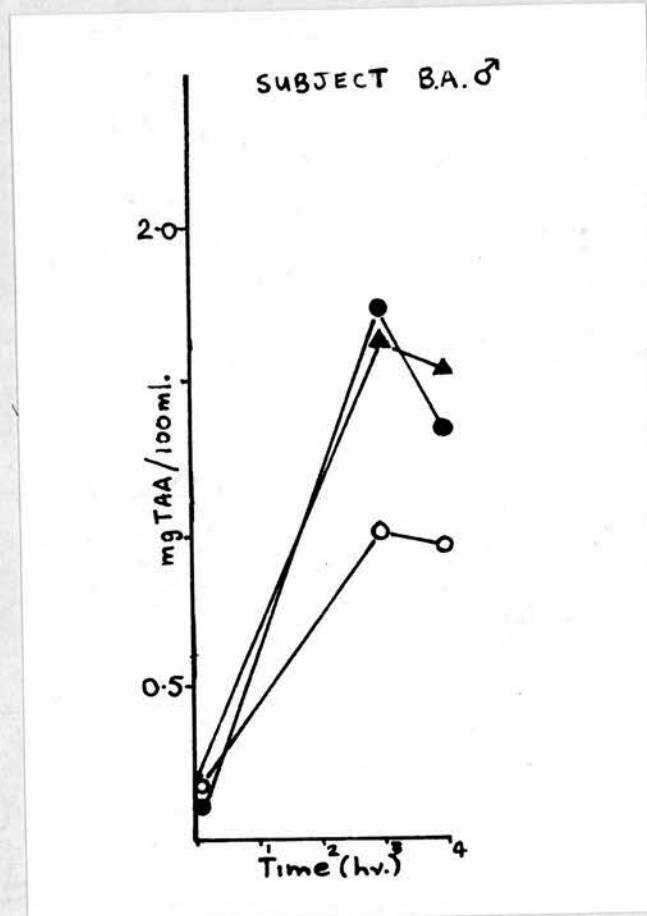
17/1 Sustained a R. pulmonary embolism - on anti-coagulants.

19/2 3rd Vit. C tolerance test.

7/2 Anti-coagulants discontinued.

12/2 Discharged - well healed wound.

FIGURE 19.



CASE 6.

Subject B.A. ♂ Age 60. Operative procedure:- Removal of Calculus.

History:- Intermittent pain L. groin for 3 years, becoming more frequent.

Relevant notes:- Appetite good.

3/1/64 Admitted.

9/1 1st Vit. C tolerance test. Hb 96%.

10/1 Operation. Anaesthetic:- Pentothal, nitrous oxide, oxygen and Halothane. Staghorn calculus removed piecemeal. Kidney approximated with interrupted mattress stitches. Abdomen closed in layers.

12/1 2nd Vit. C tolerance test. I.V. fluids stopped.

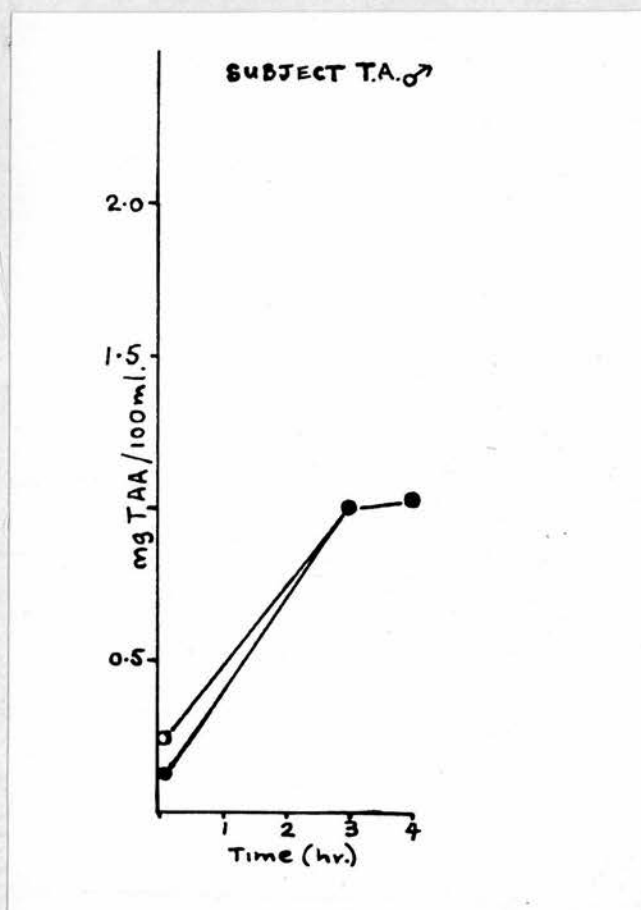
13/1 Remarkably little post op. pain.

17/1 3rd Vit. C tolerance test.

23/1 Discharged.

Post-operation recovery exceptionally good.

FIGURE 20



CASE 7

Subject T.A. ♂ Age 61. Operative procedure:- Prostatectomy.

History:- Increasing incontinence and frequency of micturition for 3 months.

Relevant notes:- Lives with wife and 7 children. Youngest 14 years old. Appetite good. Enjoys all food, particularly vegetables and fruit.

25/11/63. Admitted. General condition good. Moderate enlargement of prostate. Hb 85%.

28/11 1st Vit. C tolerance test.

29/11 Operation. Routine prostatectomy was carried out. Offending middle lobe was removed. Anaesthetic:- Pentothal, nitrous oxide, oxygen, Halothane. 12 hrs. after operation the catheter was reported as blocked which immediately required further operative procedure.

30/11 24 hrs. after operation the catheter was still unsatisfactory. Wound reopened - further investigation showed the tip of the catheter had been too short to enter bladder. Anaesthetic:- nitrous oxide, oxygen and Halothane.

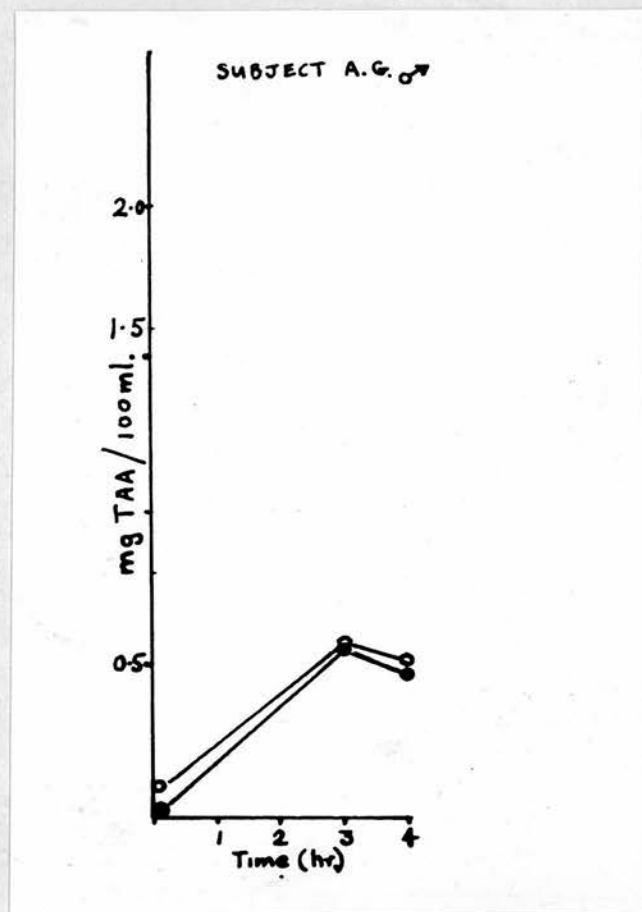
1/12 2nd Vit. C tolerance test. I.V. infusion stopped.

5/12 Condition satisfactory.

12/12 Staph. aureus in C.S.U. Started on Penbritin.

1/1/64 Recovery normal. Discharged.

FIGURE 21.



CASE 8.

Subject A.G. ♂ Age 61. Operative procedure:- Prostatectomy.

History:- 4/12/63 admitted with frequency of micturition for past 18 months. Also tired easily. Hb 72%. Poor renal function. Blood urea 131 mg.%. I.V.P. showed non-secreting kidneys on both sides.

Relevant facts: Lives with wife who has peptic ulcer. Not much fruit in house since wife dislikes it. Appetite good. Eats almost anything.

31/12/63 Cystoscopy. Moderate enlargement of prostate.

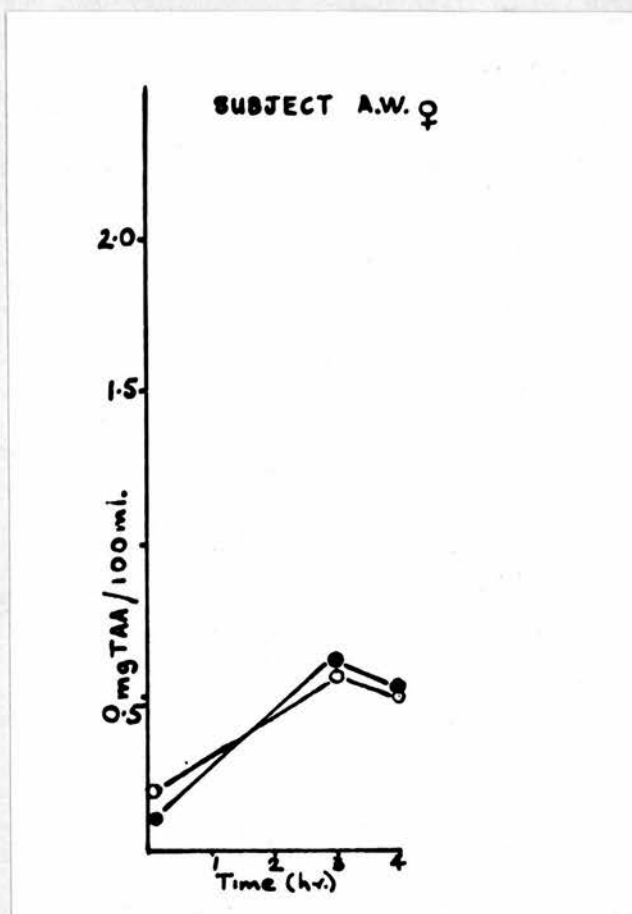
20/1/64 1st Vit. C tolerance test.

21/1 Operation. Anaesthetic:- Pentothal, nitrous oxide, oxygen, Halothane. Post-operative condition very poor.

23/1 2nd Vit. C tolerance test. Condition satisfactory.

6/2 Discharged. Blood urea 96 mg.%.

FIGURE 22.



CASE 9.

Subject A.W. ♀ Age 56. Operative procedure:- Cholecystectomy.

History:- 17/10/63. Admitted with pain in right hypochondrium, both shoulders and back. Vomiting immediately after food for 1 week. Cholecystogram showed non-functioning gall-bladder. Hb 90%.

Relevant notes:- Appetite poor. Disliked fatty foods but enjoyed fruit and fruit juices.

27/10/63 1st Vit. C tolerance test.

28/10 Operation. Cholecystectomy carried out without complications. Anaesthetic:- Pentothal, nitrous oxide, oxygen and Halothane.

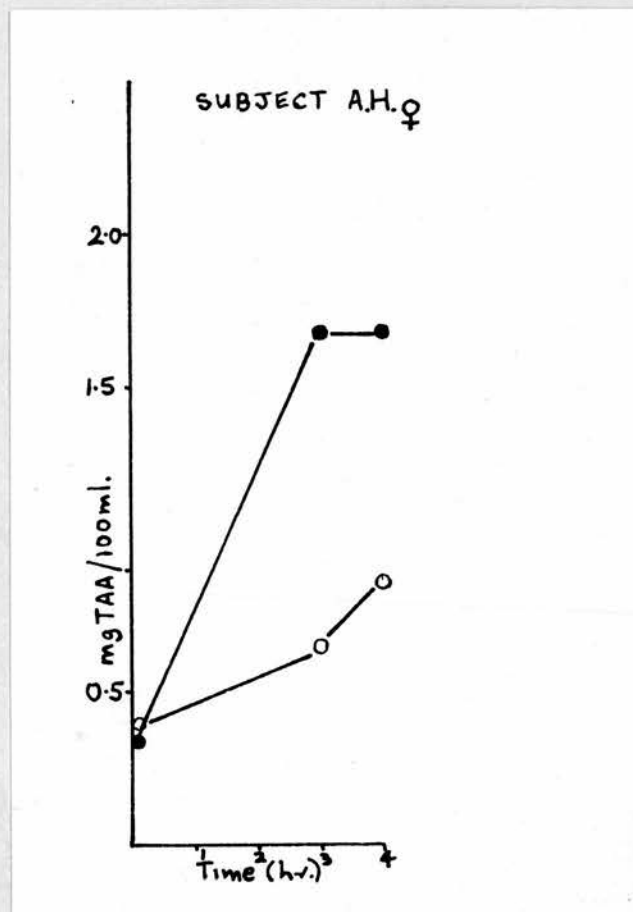
30/10 2nd Vit. C tolerance test.

2/11 Condition satisfactory.

11/11 Alternate stitches removed, others kept in longer due to patient's obesity.

14/11 Stitches out - wound normal.

FIGURE 23.



CASE 10.

Subject A.H. ♀ Age 53. Operative procedure:- Removal of renal calculus.

History:- 9/63 attended M.O.P.D. complaining of pain in left groin for 15 months with bouts of haematuria. X-ray showed a small stone present in left renal pelvis.

Relevant notes:- Appetite good.

20/11/63 Admitted.

24/11 1st Vit. C tolerance test.

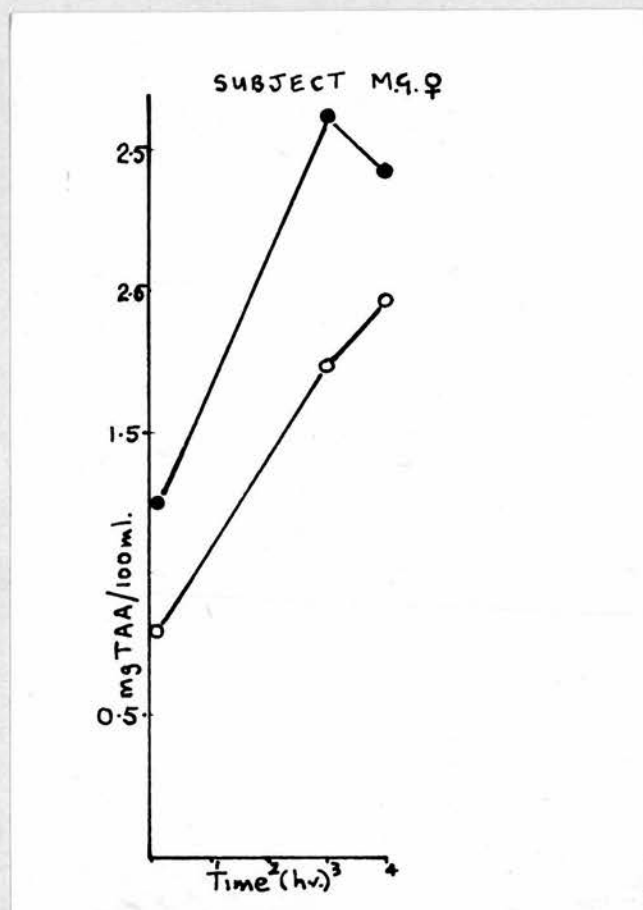
25/11 Operation. Anaesthetic:- Pentothal, Atropine, Nitrous oxide, oxygen and Halothane. Small incision made over stone which was removed with ease. Peritoneum opened inadvertently and closed. Wound in pelvis closed with three sutures and wound then closed in layers.

27/11 2nd Vit. C tolerance test.

4/12 Wound satisfactory - well-healed.

5/12 Discharged.

FIGURE 2A.



CASE 11.

Subject M.G. ♀ Age 55. Operative procedure:- Excision of cervical stump.

History:- 1959 Subtotal hysterectomy.

1960 Pelvic floor repair.

10/63 Patient found to have diabetes. This was treated and patient stabilised. Now complains of discomfort on urinating.

Relevant notes:- Appetite good.

17/12/63 Admitted for examination.

18/12 1st Vit. C tolerance test.

19/12 Operation. Anaesthetic:- Pentothal, Atropine, Pethidine. Cervical stump excised and a posterior colpoperineorrhaphy was carried out.

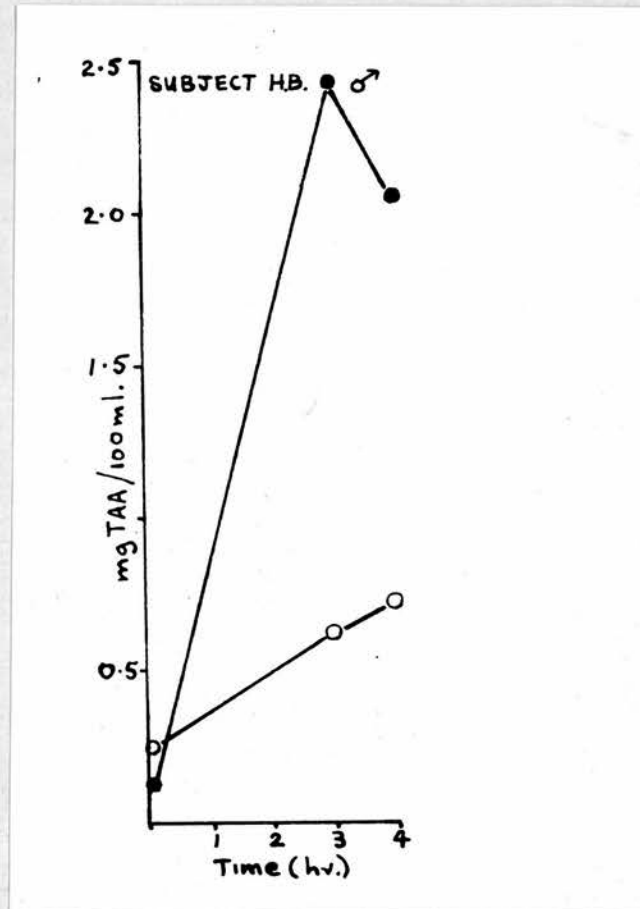
21/12 Condition good. 2nd Vit. C tolerance test.

29/12 On Terramycin 250 mg.bd.

2/1/64 No urinary symptoms - wound healed.

4/1 Discharged.

FIGURE 25.



CASE 12.

Subject H.B. ♂ Age 29. Operative procedure:- Partial gastrectomy.

History:- Duodenal ulcer for previous five years.

Relevant notes:- Appetite good normally though not recently.

25/11/63 Admitted for partial gastrectomy.

26/11 1st Vit. C tolerance test. Hb 100%.

27/11 Operation. Anaesthetic:- Pentothal. Duodenum showed anterior ulcer. High partial gastrectomy performed. Abdomen closed in layers.

28/11 Drip discontinued.

29/11 2nd Vit. C tolerance test.

3/12 Soft abdomen - feels well.

6/12 Discharged.

Vitamin C Tolerance Tests on Surgical Patients with
Simultaneous Administration of D-xylose

A series of six patients was studied using D-xylose as previously described to correct for any change in the rate of intestinal absorption after surgery. The results from these surgical patients are presented in Table 12 together with those from one normal subject.

Vitamin C Nutritional Status and Post-operative Recovery

The relationship between the vitamin C nutritional status and the course of post-surgical recovery was investigated and the results are shown in Table 13. The index used to assess nutritional status was the level of the maximum plasma concentration attained during the pre-operative tolerance test (Stotz et al, 1942).

Discussion

The significance of the normal response to a vitamin C tolerance test has already been discussed. It has been confirmed that a relationship exists between the leucocyte AA level, the time required to saturate the subject on a daily intake of 11 mg. of AA/Kg. body weight (Harris and Abbasy, 1937) and the result of the tolerance test performed as described by Dutra et al (1959) for the diagnosis of clinical scurvy (Table 10). Dutra et al stated that a rise of plasma AA in excess of 0.25 mg./100 ml. was inconsistent with a diagnosis of scurvy. Table 10 shows that five sub-

TABLE 12.

Pre- and post-surgical response to vitamin C compared by means of the xylose/ascorbate ratio, 3 and 5 hours after dosage

Patient	Operation	Blood Concentrations mg./100 ml.						Plasma Ratio of Xylose/TAA			Excretion over 5 hours of test		
		Fasting		3 hour		5 hour		3 hour	5 hour	TAA (mg.)	Xylose (gm.)	Urinary Volume (ml.)	
		TAA	XY	TAA	XY	TAA	XY						
N.B.* ♀ Age 33 ⁺	No surgical (a) procedure (b)	0.97 1.58	nil nil	2.25 2.55	7.5 8.1	1.6 2.3	2.4 2.4	3.3 3.0	1.5 1.1	44.0 54.0	2.1 2.2	600 710	
L.S. ♀ Age 46 ⁺	Hysterec- tomy	(a) 0.1 (b) 0.1	nil nil	0.36 0.34	14.2 16.0	0.34 0.23	4.0 5.3	39.4 47.0	11.7 22.6	3.0 1.0	1.2 1.5	142 156	
M.F. ♀ Age 62 ⁺	Hysterec- tomy (laparot- omy)	(a) 0.85 (b) 1.0	nil nil	1.5 1.5	9.0 10.8	1.2 1.27	4.3 5.2	6.0 7.2	3.6 4.1	1.5 10.4	1.8 2.06	460 350	
J.McL. ♀ Age 62	Amputation R. leg	(a) 0.6 (b) 0.54	nil nil	1.89 1.25	13.8 10.8	1.73 1.16	10.3 9.5	7.3 8.6	6.0 8.2	4.8 3.3	0.44 0.56	110 150	
M.R. ♀ Age 53 ⁺	Hysterec- tomy	(a) 0.12 (b) 0.06	nil nil	0.35 0.51	10.4 21.4	0.14 0.49	1.9 8.4	29.7 42.0	13.6 17.1	3.0 2.1	2.3 3.2	410 540	
J.S. ♂ Age 48	Cholecys- tectomy	(a) 0.0 (b) 0.22	nil nil	1.3 1.3	8.2 10.1	1.16 1.26	4.7 5.3	6.3 7.8	4.1 4.2	1.3 15.0	1.6 1.93	650 450	
C.B. ♂ Age 56	Prostatec- tomy	(a) 0.1 (b) 0.14	nil nil	0.85 0.37	10.3 9.8	0.8 0.28	6.9 7.1	12.1 26.5	8.6 25.3	1.0 6.4	0.26 0.57	110 640	

* Normal Response.

(a) Pre-surgical values

(b) Post-surgical values

Case reports of subjects in Table 12.

Case 13.

Subject L.S. ♀ Age 46. Operative procedure:- Total hysterectomy.

History:- Menorrhagia for previous 18 months - did not respond to Primolut N.

Relevant notes:- Appetite good.

24/1/64 Admitted. Hb 80%
26/1 1st Vit. C tolerance test.
27/1 Operation. Total hysterectomy. Anaesthetic - Pentothal.
29/1 2nd Vit. C tolerance test.
1/2 Condition satisfactory.
6/2 Wound healing well except in middle where wound did not heal by first intention.
8/2 Wound resutured under local anaesthetic.
14/2 Wound healed. Discharged home.
28/3 S.O.P.D. attendance. Wound reopened at home but has since healed well.

Case 14.

Subject M.F. ♀ Age 62. Operative procedure:- Laparotomy.

History:- Post menopausal bleeding 1/64. Complained of pain in both iliac fossae.

Relevant notes:- Appetite good. Likes vegetables but not fruit.

8/2/64 Admitted.
9/2 1st Vit. C saturation test.
10/2 Operation. Anaesthetic: pentothal. Laparotomy was carried out which showed multiple nodules throughout the peritoneal cavity. Condition considered inoperable.
12/2 2nd Vit. C tolerance test.
15/2 Wound healing normally.
25/2 Discharged.

Case reports of subjects in Table 12.

Case 15.

Subject J.McL. ♀ Age 62. Operative procedure:- Amputation.

History:- Complained of intermittent claudication of both legs for previous ten years. Amputation of R. leg above knee in November 1962 for arterio-sclerotic gangrene. Gangrene developed in L. foot on 4/2/64.

Relevant notes:- Lived alone. Appetite good, but lost interest in cooking. Had kind neighbours who often cooked for her. Liked vegetables but not fruit.

17/2/64 Admitted. Hb 80%.
20/2 1st Vit. C tolerance test.
21/2 Operation. Anaesthetic: pentothal. Amputation of L. leg above knee.
23/2 2nd Vit. C tolerance test.
25/2 Drain removed.
28/2 Hb 65%. Started on iron and vit. C (50 mg. t.i.d.).
4/3 Sutures removed. Wound satisfactory.

Case 16.

Subject M.R. ♀ Age 53. Operative procedure:- Total hysterectomy.

History:- Removal of renal calculi when aged 30.
Removal of vaginal cyst when aged 46.
1/64. Complained of irritation and referred to gynaecologist who found ovarian cyst and senile vaginitis.

Relevant notes:- Appetite good, no loss of weight or anaemia.

3/3/64 Admitted. Hb 78%.
4/3 1st Vit. C tolerance test.
5/3 Operation. Anaesthetic: pentothal, cyclopropane and oxygen. Total hysterectomy.
7/3 2nd Vit. C tolerance test.
8/3 Condition satisfactory.
10/3 Wound healed normally. Patient up and about. Hb 62%.
20/2 Discharged to convalesce with well-healed wound.
~~Discharged to convalesce with well-healed wound.~~

Case reports of subjects in Table 12.

Case 17.

Subject J.S. ♂ Age 48. Operative procedure:- Cholecystectomy.

History:- Complained of pain below ribs on right costal margin for a month before admission. Treated for flatulence.

Relevant notes:- Diet moderate for previous month.

7/3/64 Admitted with pain in R. upper abdomen and back.
12/3 1st Vit. C tolerance test.
13/3 Operation. Anaesthetic: pentothal, pethidine. Cholecystectomy.
No complications.
14/3 Slight pyrexia.
15/3 2nd Vit. C tolerance test.
18/3 Normal healing of wound.
21/3 Discharged.

Case 18.

Subject C.B. ♂ Age 56. Operative procedure:- Prostatectomy and excision of bladder diverticulum.

History:- Frequency of micturition for 10/12.

Relevant notes:- Lives at home, appetite good.

17/3/64 Admitted.
19/3 1st Vit. C tolerance test.
20/3 Operation. Anaesthetic: pentothal, nitrous oxide and halothane.
Prostatectomy and excision of calculus in infected diverticulum.
21/3 Pyrexia.
22/3 2nd Vit. C tolerance test.
23/3 Wound - redness round stitches.
27/3 Grade 2 wound infection, and chest infection. Start Furadantin
100 mg.
1/4 Discharged.

Terms of Reference for Classifications Shown in Table 13

Vitamin C Nutritional Status

The status of the patient was rated from the value of the maximum plasma level attained during the first tolerance test. (Stotz et al, 1942)

<u>Maximum plasma level</u>	<u>Status rating</u>
under 0.5 mg.%	Poor
0.5 - 1.0 mg.%	Medium
1.0 - 1.5 mg.%	Good
over 1.5 mg.%	Very good

Post-operative recovery

All information was obtained from the case notes after the convalescence or discharge of the patient.

<u>Post-operative condition of wound</u>	<u>Rating</u>
Sanguinous oozing from the wound or other evidence of weakness.	Poor
Uneventful convalescence with normal healing rate	Good
Exceptionally rapid healing	Very good

TABLE 13

Relationship between vitamin C nutritional status and post-operative recovery.

Case No.	Figure or Table No.	Subject	Vitamin C Nutritional Status Prior to Surgery	Post-operative recovery
1	Fig. 14	R.S.	Medium	Poor
2	15	W.W.	Very good	Very good
3	16	R.A.	Good	Good
4	17	D.O.	1st operation * - 2nd operation Near scorbutic	Very poor Very good (after saturation with vitamin C)
5	18	A.B.	Very good	Good
6	19	B.A.	Very good	Very good
7	20	T.A.	Good	Good
8	21	A.G.	Medium	Poor
9	22	A.W.	Medium	Good
10	23	A.H.	Very good	Good
11	24	M.G.	Very good	Good
12	25	H.B.	Very good	Good
13	Table 12	L.S.	Medium	Poor
14	"	M.F.	Good	Good
15	"	J. McL.	Good	Good
16	"	M.R.	Medium	Good
17	"	J.S.	Good	Good
18	"	C.B.	Good	Good

* In this case the first operation referred to was the amputation of left leg on 3/63, (see case note). It is probable that the poor vitamin C status, discovered before the second operation, also existed before the first and was responsible for the very poor post-operative recovery on that occasion.

jects were scorbutic according to this criterion, and the highest leucocyte AA level amongst this group of five was 66 $\mu\text{g.}/10^9$ cells (7.4 mg./100 ml.). This figure agrees with the findings of Crandon et al (1961) who observed a higher rate of post-surgical wound dehiscence among subjects with white cell AA values less than 8 mg./100 ml. (70 $\mu\text{g.}/10^9$ cells). The present work showed that if the leucocyte AA content was above 70 $\mu\text{g.}/10^9$ cells, the tolerance test was normal (non-scorbutic), and normal curves were also obtained together with high white cell AA values, from all patients when saturated with the vitamin.

Although the number of days required to saturate these patients with vitamin C gives some indication of the degree of deficiency present, the lack of close correlation between the time taken to saturate and the leucocyte level of AA confirms the criticisms made against such saturation tests by Bartley, Krebs and O'Brien (1953) and by Sinclair (1948). These authors stressed that without a full knowledge of the metabolism of the vitamin, and in the absence of understanding about the mechanism of its renal excretion, the appearance of vitamin C in the urine after a test dose cannot be assumed to give reliable information regarding tissue stores.

The geriatric tolerance tests (Table 11) indicated that these elderly people were deficient in vitamin C. Four

out of ten had scorbutic-type curves, though none showed any clinical signs of scurvy. These elderly subjects, who had for a long time been receiving a low intake of vitamin C, could not be distinguished from true scorbutics either by leucocyte AA determinations or by their response to a tolerance test. In the writer's opinion such subjects were latent scorbutics and would have manifested deficiency symptoms in the event of physical trauma.

As already stated in Part II, a series of leucocyte AA determinations on elderly orthopaedic cases was planned but could not be carried out for reasons already mentioned. The leucocyte AA levels of this orthopaedic group were so low that, like the deficient geriatric subjects in Table II, they were probably latent scorbutics. In elderly patients, Bourne (1944) showed that, if poor callus formation followed the occurrence of fracture, it had the effect of retarding bone formation. Deficiency of vitamin C might well be an important factor contributing to the prolonged periods often required for fractures to heal in elderly subjects whose economic status is poor.

The interpretations of Figures 14 - 25 will now be discussed. Figures 14 - 19 show increased post-surgical use of vitamin C though the near-scorbutic type of curve found pre-operatively in Figure 17 (Subject D.O.) almost obscures the post-surgical depression of the plasma TAA.

Figures 18 and 19 show the effect of the continuation of trauma and the recovery from surgical trauma respectively. This increased vitamin C requirement in post-surgical patients might depend on the hormones of the adrenal cortex. Crandon et al (1961) showed that the effects of surgery on leucocyte and plasma AA could be simulated by the administration of ACTH or cortisone and concluded that these agents caused a change in the distribution of AA in the body rather than an alteration in its metabolism.

No alteration in vitamin C requirement after surgery can be demonstrated in Figures 20 - 22. Even in these cases, a suggestion that the use of the vitamin is increased after surgery may be deduced from the failure of the second tolerance test to show increased plasma concentrations of TAA relative to the first; such increases are demonstrated in the normal response (Figure 13).

The interpretation of Figures 23 - 25 is uncertain due to the existence of alternative explanations for the different shape of the post-surgical curves. Additional information is necessary in these types of cases regarding relative intestinal absorption rates, before it can be decided if the occurrence of a later maximum in the second curve is due to delayed absorption from the intestine, or to a combination of this and an increased cellular absorption from the plasma.

The results of Table 12 show that the use of D-xylose in these studies helps considerably in the interpretation of results. At the top of this table are shown the results of a test carried out on a normal individual; these show that the three and five hour xylose/ascorbate ratios had decreased in the second tolerance test. In each of the six surgical cases presented, increased values of xylose/ascorbate ratios could be demonstrated. In three of these cases (J.M., C.B. and I.S.) the curve performed after surgery had lower values than the pre-surgical one, an observation similar to the findings in Figures 14 - 19. In cases M.F. and J.S., increased ratios were demonstrable even when the successive tolerance tests showed no alteration in requirement (cf. Figures 20 - 22). In subject M.R. the increased ratio observed after surgery was seen even in the presence of a normal increase in the plasma concentration of the vitamin during the second test; this was interpreted as being due to a much more rapid intestinal absorption in the post-operative period, the evidence for this being the increase in plasma xylose concentration.

The five hour urine loss of TAA and xylose was also determined, and Table 12 shows that in no case was the urinary loss of TAA sufficient to influence the increase in value of the xylose/ascorbate ratio, except in the normal subject (N.B.). In the normal individual the effect of this

was to minimise the decrease in xylose/ascorbate ratio, which would otherwise have been much smaller on the second occasion because the plasma concentration of TAA would not have been limited by the increased diuresis in the second test.

Summary of Results

1. Increases have been shown in the metabolic requirement of vitamin C due to surgical trauma. These increased requirements have been demonstrated by means of plasma xylose/ascorbate ratios.
2. Many elderly people are deficient in vitamin C. On the basis of findings described in this section they would have an unfavourable prognosis after major surgery, unless given large supplements of the vitamin.

Intestinal Absorption of Vitamin C

The increase in the blood level of AA observed several hours after ingestion of vitamin C occurs whether the vitamin is given in the oxidised or the reduced form (Linkswiler, 1958; Sabry and Dodds, 1958; Sabry, Fisher and Dodds, 1958). However, the form in which vitamin C is absorbed from the intestine has not been satisfactorily established, although some interesting observations can be made which will now be discussed.

As shown in this thesis, when an oral dose of 6 mg AA/Kg. body weight (Approx. 420 mg. total dose for an average adult) is given, the maximum plasma concentration is reached in about three hours. Thomson (1955) showed the maximum blood concentration occurred only 30 mins. after an oral dose of 450 mg. DHA. This difference suggests either that both forms are assimilable, but the oxidised more readily, or that DHA only can be taken up, the speed of absorption of AA being then limited by the rate of its intestinal oxidation.

On the analogy of the behaviour of the leucocyte, the second possibility seems the more likely. According to this hypothesis, DHA would be taken up by the cells of the intestinal wall, reduced to AA, and then passed into the general circulation. The fact that the vitamin can be detected in the reduced form during absorption (Thomson,

1955; Dutra et al, 1959) has been confirmed in the present work, and it seems unlikely that the reduction of DHA in the blood stream is efficient enough to allow it to be absorbed from the intestine and yet prevent DHA from being detected in blood collected from an arm vein.

It has been shown in this thesis that DHA can penetrate the leucocyte membrane whereas AA cannot. It has also just been suggested that DHA is the form in which the vitamin enters the intestinal wall. These observed facts and proposals support the opinion of Martin (1961) who pointed out that DHA was not an acid and therefore was unionised; this renders DHA diffusible whereas AA is the undiffusible form of the vitamin.

However, these facts cannot explain every observation. AA can be transported across cell boundaries in the reduced condition in some tissues (Langham, 1958). Langham showed that AA, but not DHA, would pass from the plasma to the anterior and posterior aqueous humours of the eye. It would also appear that AA, but not DHA, is involved in renal tubular absorption of Vitamin C (Knox and Goswami, 1961), since the reduced form of the vitamin is completely filterable by the renal glomerulus (Sargent and Golden, 1951). In addition, indirect evidence for the existence of a mechanism for the absorption of AA by some cell species is provided by the results of the AA tolerance test on scorbutics (Dutra

et al., 1958); in whom the test dose fails to produce an increase in plasma levels. It has been shown in the present work that the test dose appears in the blood in the form of AA, and it seems likely therefore that somewhere in the body there are structures capable of removing the reduced vitamin from the blood stream.

The findings of Kanungo and Patnaik (1964) support the existence of a mechanism for the absorption of AA. ~~somatic structures~~. These authors demonstrated the uptake of the reduced vitamin by skin and bone marrow in young rats, and showed that this uptake decreased to some extent with age.

Increased Requirements of Vitamin C in Surgical Patients

Increased use of vitamin C has been shown to occur after surgical procedures. This increase was demonstrated 48 hours after surgery in the present studies, and immediately following operation by Crandon et al (1961). It can be concluded, therefore, that the increased requirement of surgical patients for vitamin C occurs subsequent to operation and persists for at least 48 hours. The present thesis also indicates that, if the trauma associated with surgery continues, an increased requirement for the vitamin persists. It has been suggested that hormones of the adrenal cortex might be involved in this altered pattern of vitamin C metabolism, but this view is not held by Kark (1953).

Grandon et al (1961) have stated that an intake of 300 mg./day is sufficient to meet all the demands of the post-surgical patient, and Coon (1962) has shown that a higher level of intake than usual is necessary to achieve saturation in surgical patients and he concludes that 200 mg. of vitamin C per day is advisable in a surgical population. No quantitative assessment of the vitamin requirement of such subjects has been attempted in the present work, but certain observations can be made in this respect. The tolerance tests summarised in Table 12 and the tests shown in details in Figures 14 - 25 were carried out using loading doses of about 0.5 g. vitamin C (6 mg./Kg. body weight); this dose would normally produce a saturation response immediately. The results on surgical patients, however, show that they would require several times this dosage to produce the same effect. This proportional increase in requirement for vitamin C over the normal subject suggests that 200 - 300 mg. of the vitamin would be a suitable daily level of intake after surgery.

Elderly subjects are different in this respect. As has been shown in the present studies, they probably require larger initial supplements of the vitamin to produce high levels in the tissues, and then need to be maintained on the suggested daily intake. The rapidly growing problem of the treatment and care of the geriatric population at

the present time is giving concern. Considering the large proportion of orthopaedic cases which occur in geriatric patients, the principles of preventive medicine, as they were applied so long ago in relation to vitamin C by Lind (1753), could well be applied to these cases. The place where these principles could best be applied would require the co-operation of the social service workers of the Women's Voluntary Service, who supply meals to the aged and infirm. The preoccupation of "meals on wheels" with serving hot food unfortunately overlooks the extreme lability of vitamin C under these conditions, and renders the main meal of the day, quite worthless for these elderly people in respect of this indispensable accessory factor.

Although Crandon et al (1961) showed a greater incidence of wound dehiscence among subjects having leucocyte levels below 8 mg./100 ml. ($70 \mu\text{g.}/10^9$ cells), it has not been demonstrated unequivocally that post-operative recovery is benefited by maintenance of tissue saturation. However, in support of high nutritional intakes the metabolic defect in tyrosine metabolism induced by oral doses of this amino acid, can only be demonstrated in subjects whose tissues are less than saturated with vitamin C (Knox and Goswami, 1961), which suggests that when dietetic supplies of an accessory food factor are not sufficient for current needs the chemical processes within cells are impaired long before

physical manifestations of deficiency appear (Sebrell and Harris, 1954).

As well as the primates and the guinea pig, two further species, a fruit-eating bat and a bird, the red-vented bulbul (Roy and Guha, 1958), have lost the ability to synthesise vitamin C and are therefore dependent on dietary supplies of this vitamin. In each of these animals, the biochemical lesion in their intermediary metabolism has been shown to be lack of the enzyme L-gulonolactone oxidase (Chatterjee, Kar, Ghosh and Guha, 1961). This enzyme catalyses the conversion of L-gulonolactone, itself formed from L-gulonic acid in the glucuronic acid cycle, to 2-keto-L-gulonolactone which enolises to AA. The tissues of animals possessing L-gulonolactone oxidase are in a state of permanent saturation with respect to vitamin C. It could be argued from this, teleologically, that man's ideal intake should be one which will just produce a state of saturation. This would cover all needs in any eventuality, in health or disease and avoid any risk that in a particular circumstance his intake of the vitamin would be sub optimal.

General Summary

1. DHA is absorbed and reduced to AA by human leucocytes. In contrast AA is not absorbed.
2. Only the reduced form of vitamin C is found within the leucocyte.
3. Leucocyte AA diffuses slowly to the surrounding medium.
4. Entry of DHA into and its subsequent reduction by leucocytes are independent processes.
5. Sulphydryl compounds are involved in the leucocytic reduction of DHA.
6. The concentrations of GSH and AA in the leucocytes in acute myeloid leukaemia are below the normal ranges for these compounds.
7. A normal range of leucocyte AA has been given.
8. Many elderly people are deficient in vitamin C. This has been shown by their response to vitamin C tolerance tests and by the occurrence of very low leucocyte AA concentrations. It has been suggested that geriatric patients require supplements of the vitamin routinely before and particularly after surgery.
9. Increased requirements of vitamin C in the 48 hours following surgical trauma have been demonstrated. Thereafter the increased metabolic use of vitamin C decreases unless complications adding to the trauma occur.

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APPENDIX I

<u>Authors</u>	<u>Ranges of Leucocyte Ascorbic Acid in Normal Subjects</u>	
	<u>mg./100 ml.</u>	<u>μg/10⁹ cells</u>
Butler, Cushman and MacLachlan, (1943).	22 - 36	(197 - 322)
Tourney, Chevillard and Hamon, (1943).	23 - 30	(206 - 268)
Lowry, (1946).	11.9 - 23.7	(107 - 212)
Bodansky, Wroblewski and Markardt, (1952).	22.5 - 51.5	(202 - 463)
Crandon, Mikal and Landau, (1952).		
* Oct.	10 - 50	(89 - 448)
* Feb.	4.1 - 25	(37 - 224)
Overall range	4.1 - 50	(37 - 448)
Waldo and Zipf, (1955).	21.8 - 52.0	(195 - 468)
Barkhan and Howard, (1958).	(12.1 - 25.1)	108 - 224
Denson and Bowers, (1961).	(23.4 - 59.2)	210 - 530
(Subjects aged 65-91)	(2.3 - 40.0)	20 - 360
Present Thesis (Subjects aged 18-38)	(15.0 - 38.4)	135 - 343
If elderly subjects are included the overall range extends down to the limits of detection.	(2.3 - 38.4)	20 - 344

The ranges in brackets have been calculated from the figures in the adjacent column which were obtained by direct measurement by the authors concerned.

* Crandon et al (1952) showed an interesting seasonal variation in the leucocyte levels of vitamin C in normal subjects.

APPENDIX II.

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**The uptake and reduction of dehydroascorbic acid
by human leucocytes**

It has been reported that ascorbic acid added to whole blood¹ or to leucocyte suspensions in various media^{2, 3} is taken up by the leucocytes. LLOYD^{4, 5} on the other hand found that an increase in the total ascorbic acid content of leucocytes followed the addition of dehydroascorbic acid, but not of ascorbic acid, to defibrinated blood. If, however, he removed the erythrocytes before adding ascorbic acid, it accumulated in the leucocytes.

Since ascorbic acid is readily oxidised to dehydroascorbic acid and since erythrocytes readily reduce dehydroascorbic acid⁶, it seemed worthwhile to reinvestigate the uptake of ascorbic acid and dehydroascorbic acid by leucocytes measuring separately (reduced) ascorbic acid and total ascorbic acid.

Saline suspensions of normal human leucocytes were prepared by differential centrifugation of leucocyte-rich plasma⁷. Ascorbic acid or dehydroascorbic acid was then added to give a final concentration of 5 mg per 100 ml. Dehydroascorbic acid was prepared immediately before use by the method of PATTERSON⁸. The ascorbic acid content of metaphosphoric acid filtrates of the leucocyte suspensions was determined according to OWEN and IGGO⁹. The total ascorbic acid (ascorbic acid, dehydroascorbic acid and diketogulonic acid) in trichloroacetic acid filtrates of the suspensions was estimated by the method of ROE AND KUETHER¹⁰. A Coulter electronic counter was used for the enumeration of leucocytes¹¹.

Table I shows the results in a typical experiment. Dehydroascorbic acid is rapidly taken up by the cells and is there completely reduced to ascorbic acid, but there is no demonstrable uptake of ascorbic acid itself within the experimental period of 30 min.

Oxidation of ascorbic acid to dehydroascorbic acid by molecular oxygen takes place readily. It seems thus likely that previous workers^{1-3, 5} observed the uptake not of ascorbic acid, but of dehydroascorbic acid formed from it during their experiments.

TABLE I
UPTAKE OF DEHYDROASCORBIC ACID BY LEUCOCYTES

Exposure time min	Cells exposed to dehydroascorbic acid		Cells exposed to ascorbic acid	
	Ascorbic acid	Total ascorbic acid	Ascorbic acid	Total ascorbic acid
0	140	150	135	150
15	250	250	140	140
30	320	330	140	140

Results expressed as cellular content of ascorbic acid ($\mu\text{g}/10^9$ cells) after exposure to ascorbic acid or dehydroascorbic acid (50 $\mu\text{g}/\text{ml}$) in physiological saline at room temperature. 16,000 cells/ μl of suspension.

There is no need to postulate an active transport mechanism for dehydroascorbic acid since, as we have shown, it is completely reduced to ascorbic acid on entry into the cell. MARTIN¹² has recently pointed out that dehydroascorbic acid is unionised and so is more diffusible at body pH than is the negatively charged ascorbate ion. He suggests that free diffusion of dehydroascorbic acid into cells followed by intracellular reduction to the less diffusible ascorbate ion could explain the normal occurrence of higher concentration of ascorbate in leucocytes than in plasma.

In the course of this work, we have found that there is a slow release of ascorbic acid from leucocytes into the suspending medium over a few hours, that dehydroascorbic acid is not reduced by lysed leucocytes, and that both uptake and reduction of dehydroascorbic acid by leucocytes are inhibited by *p*-chloromercuribenzoate or iodoacetate.

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